AD_____

Award Number: DAMD17-98-1-8089

TITLE: Targeting of Oncogenic Proteins for Intracellular Degradation

PRINCIPAL INVESTIGATOR: Stephen W. Byers, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Final (1 Jul 1998 - 30 Jun 2002)

4. TITLE AND SUBTITLE
Targeting of Oncogenic Proteins for Intracellular Degradation

5. FUNDING NUMBERS
DAMD17-98-1-8089

6. AUTHOR(S)
Stephen W. Byers, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

E-Mail: byerss@georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

β-catenin, is an important component of the wnt signaling pathway and IκBα is an important regulator of the NF-κB pathway. Both proteins are phosphorylated at serines in the N-terminal region, which subsequently target them for ubiquitination by the same ubiquitin ligase complex. In the first year of the award, we demonstrated proof of principle that small peptides could be constructed that would enter cells and target oncogenic proteins, such as β-catenin and erbB2 for intracellular degradation. During the course of this work, we discovered that he similarities in the regulation of β-catenin and IκBα ubiquitination extended to the kinases that are involved in control of their phosphorylation. The IKK complex is responsible for the phosphorylation of IκBα while GSK-3β is thought to regulate β-catenin phosyphorylation. This work showed for the first time that IKK also exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling. Consistent with this, we found that cytokines, such as TNFα also markedly regulated β-catenin activity. Our demonstration that cytokine and β-catenin signaling are cross-regulated at the level of targeted protein degradation is important and points to a clinically significant relationship between inflammatory responses and oncogenic activity.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer	95
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS:

Introduction:

Selective depletion of intracellular oncogenic proteins is a potentially powerful tool for the treatment of breast cancer. This is usually achieved by genetic manipulation of the target gene using procedures such as gene disruption, antisense or ribozyme technologies. In the first part of this grant we proposed an alternative approach in which an oncogenic protein, in this case, β-catenin or erbB2 is specifically targeted for intracellular degradation. In order to do this we took advantage of the permeability properties of the third helix of the antennapedia protein. This was used to deliver a small trifunctional peptide consisting of a target protein binding peptide and a peptide designed to interact with the E2 class of ubiquitin conjugating enzymes. In this way we wanted to demonstrate that the ubiquitin-conjugating machinery will be selectively recruited to the target protein which should then be degraded by the proteosome. We used the cytoplasmic signaling molecule β-catenin as one model system since its oncogenic activity is regulated at the level of protein stability and we have established that it is normally targeted for ubiquitination and proteosomal degradation. Mutations of β-catenin that increase its protein stability are oncogenic. A second target was ErbB-2 a tyrosine kinase strongly associated with breast cancer. We made an ErbB-2 binding peptide consisting of the SH2 domain of grb2 which can only bind tyrosine phosphorylated ErbB-2. We showed proof of the principle that direct targeting of oncogenic proteins for intracellular degradation is feasible and could lead to the development of novel therapuetic strategies based on this approach.

In the second part of the award period we proposed to investigate the role of the IKK complex in the regulation of β-catenin phosphorylation, degradation and signaling. The IKK is the complex that targets the inhibitor of NFκB (IκB) for degradation. NFκB is a major regulator of cell death and many studies point to a role for NFκB regulation by cytokines in breast cancer. We demonstrated that cytokine activation of the IKK also regulates β-catenin phosphorylation, degradation and signaling, showing for the first time that these two pathways interact.

Methods and Results

Since this grant was first submitted we gathered important new information relating to the sites on β-catenin that regulate its phosphorylation, ubiquitination and degradation. In particular we found that IKK, a kinase thought previously to only phosphorylate IκB proteins to regulate NF kappa B, is a potent and important kinase in the regulation of β-catenin phosphorylation and ubiquitination. The results raise the possibility that vectors designed to target β-catenin may also interfere with NF-kappa B signaling. The reviewer of year 2 of the Progress Report of my grant DAMD-98-1-8089 suggested that I provide a revised statement of work to more adequately incorporate the new data on I kappa B kinase. This was duly submitted and approved.

Revised Statement of Work

Year 1

a) Construction of at least 6 expression vectors containing erbB2 and β-catenin targeting, antennapedia and cyclin B destruction box fusion constructs. These will consist of vectors containing several different erbB-2 and β-catenin targeting sequences as well as cyclin B destruction boxes of various lengths and sequence. Our aim is to ascertain the minimum size of the final trifunctional peptide product that is effective in targeting erbB-2 and β-catenin for degradation.
b) Co-express the vectors with erbB-2 or stable β-catenin (S37A mutant), investigate interaction with β-catenin.

Results year 1:

In the first annual report we described in detail the progress we had made in constructing targeting vectors for ErbB-2 and β-catenin. Please refer to this report for figures and detailed methodology. Briefly the key research accomplishments of the first 12 months were:

1) A number of different targeting vectors were constructed.
2) The targeting constructs were transfected into three different cells of varying ErbB-2 status
3) Two of the constructs yield protein products of the predicted size indicating that the recombinant peptides are stable and can be expressed at relatively high levels.
4) The constructs were detected with an antibody directed at the FLAG tag indicating that it is accessible and does not interfere with protein production.

Year 2

a) Investigate the role of the IKK complex in the regulation of β-catenin protein levels and signaling.
b) Continue experiments with expression vectors. Monitor β-catenin ubiquitination and degradation.
c) Adherex to make membrane permeant peptides and/or production of recombinant peptides.
d) Test the ability of peptides to enter cell cytoplasm and/or nucleus.

Years 3 and 4

a) Identify IKK phosphorylation sites on β-catenin.
b) Characterize the mechanism of IKK regulation of β-catenin signaling activity.
c) Redesign the targeting peptides based on this information.
d) Test the activity of the redesigned peptides on β-catenin for ubiquitination and degradation in vitro.
e) Test the ability of peptides to inhibit growth and colony formation of β-catenin-transformed cells.

Results year 2-4:

In the second half of the grant we concentrated on β-catenin. Since this grant was first submitted we gathered important new information relating to the sites on β-catenin that regulate its phosphorylation, ubiquitination and degradation. In particular we found that IKKα, a kinase thought previously to only phosphorylate IκB proteins, is a potent and important kinase in the regulation of β-catenin phosphorylation and ubiquitination (see reports for years 2 and 3). This work was summarized in previous Progress Reports and has now been published in two papers (Verma et al, 2001, Albanese et al, 2003). Another paper has also been submitted on this topic (Teo et al, 2004) see appendix). We continue to work on the targeting concept and have redesigned our strategy to take into account recent advances in the regulation of β-catenin and erbB2 degradation. Nobel Laureate, Harold Varmus recently published a paper in which he showed (using a somewhat different strategy) that targeted degradation of β-catenin protein could be achieved in vitro and in vivo (Cong et al, (2003) A protein knockdown strategy to study the function of beta-catenin in tumorigenesis. BMC Mol Biol. 4:10.). This is clearly an area of great potential and although we have not completed all the experiments outline in the SOW in this area this project is ongoing and publications that arise from it will cite this award. It is important to note that our results related to cytokine signaling and β-catenin degradation had an important bearing on the design of the next generation of targeted degradation vectors. However, our results and those of Dr. Varmus have clearly established the "proof of principle" of our approach.

Key Research Accomplishments:

1. Demonstration of proof of principle that bifunctional peptides that target oncogenic proteins for degradation can be made and can enter cells.
2. Demonstration that phosphorylation of β-catenin serine residues 33 and 37 targeted the protein for ubiquitination and subsequent degradation. This work formed the basis for many other studies from other laboratories that elucidated the nature of the SCF complex required for coupling phosphorylated β-catenin (and IκB) to the ubiquitination machinery.
3. The first demonstration that β-catenin was in fact oncogenic and regulated contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell-cycle arrest.
4. The first demonstration that a kinase, other than GSK-3, was involved in β-catenin phosphorylation.
5. The first demonstration that significant cross-regulation of β-catenin and NFκB signaling pathways occurs through the IκB kinases.
6. Cyclin D1 is required for PI3K-dependent S-phase entry in primary cells.
7. PI3K-induction of cyclin D1 is dependent upon IKKα.
8. IKKα, but not IKKβ induces cyclin D1 through β-catenin/Tcf
9. IKKα associates with and phosphorylates β-catenin and changes β-catenin signaling.
10. TNFα regulates β-catenin signaling activity

Reportable Outcomes:

Papers:

Albanese R., Jarrett, C. Joyce, D. Hughes, J. Wu, K. D'Amico, M. Fu, M. Backer, JD. Ben Ze'ev, A. Downward, J. Lamberti, C. Lin. K-M. Gaynor, RB. Byers, S. and Pestell, R. (2003) IKK regulation of cyclin D1 is mediated by changes in β-catenin signaling. Mol Biol Cell. 14: 585-599

Lamberti, C., Lin, K-M., Yamamoto, Y., Verma, IM., Byers, S. and Gaynor, R. (2001) Regulation of β-catenin function by the IκB kinases J Biol Chem. 276:42276-42286.

Orford, K., Orford, C. and Byers, S. (1999) β-catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell cycle arrest. J.Cell Biol. 146: 855-867

Easwaran,V. Song, V., Polakis, P. and Byers, S. (1999) The ubiquitin-proteosome pathway and serine kinase activity regulate APC modulation of β-catenin/LEF signaling. J. Biol Chem 274: 16641-16645.

Grants:

IKK and β-catenin in breast cancer. DAMD-17-01-1-0248. Pre-doctoral fellowship award for Marissa Teo

Degrees supported by DAMD17-98-1-8089:

Keith Orford MD.PhD 2001
Christy Jarrett PhD 2001
Vijay Easwaran PhD 1999
Marissa Teo-current student

Personnel supported by DAMD17-98-1-8089:

Not all of these personnel were paid by this grant but they were supported by supplies provide by the award.

Keith Orford MDPhD
Christy Jarrett PhD
Stephen Byers PhD
Fadwa Attiga PhD
Marissa Teo

Conclusions:

In the first part of the award period we showed proof of the principle that direct targeting of oncogenic proteins for intracellular degradation is feasible and could lead to the development of novel therapeutic strategies based on this approach. This work is ongoing with a second generation of targeting vectors which take into account our finding that there is cross-regulation of wnt/β-catenin and TNF/NFkB pathways being developed. The wnt/β-catenin and NFκB pathways regulate the transcription of genes that are involved in cell cycle control and cellular differentiation. In addition the NF-κB pathway is involved in the induction of the inflammatory response. β-catenin, a known oncogene, is an important component of the wnt signaling pathway and IκBα is an important regulator of the NF-κB pathway. Both proteins are phosphorylated at serines in the N-terminal region, which subsequently target them for ubiquitination by the same ubiquitin ligase complex. In the first year of the award we demonstrated proof of principle that small peptides could be constructed that would enter cells and target oncogenic proteins, such as β-catenin and erbB2 for intracellular degradation. During the course of this work we discovered that the similarities in the regulation of β-catenin and IκBα ubiquitination extended to the kinases that are involved in control of their phosphorylation. The kinases that are important in the phosphorylation of these proteins have been intensely studied. The IKK complex is responsible for the phosphorylation of IκBα while GSK-3β is thought to regulate β-catenin phosphorylation. The IKK complex contains two kinases, IKKα and IKKβ. Gene disruption studies in mice indicate that IKKβ is the dominant component of the IKK complex involved in phosphorylation of IκBα. The work funded by this grant showed for the first time that IKK also exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling in APC-mutant colon cancer cells with high endogenous β-catenin levels. Consistent with this we found that cytokines, such as TNFα also markedly regulated β-catenin activity. Our demonstration that cytokine and β-catenin signaling are cross-regulated at the level of targeted protein degradation is important and points to a clinically significant relationship between inflammatory responses and oncogenic activity.

Appendices (manuscripts and papers not previously submitted)

Teo, M, Attiga, F. Jarrett, C, Zipper, L, Verma, U, Gaynor, R, Pestell, R. and Byers, SW. TNFα regulation of β-catenin signaling. Submitted

Submitted MS Appendix 1

TNFα Regulation of β-catenin Signaling

[†]Marissa Teo, [†]Fadwa Attiga, [†]Christy Jarrett, [†]Laurie Zipper, [†]Todd Waldman, *Inda Verma, *Mark Schmidt, [+]Udit Verma,[†]Richard Gaynor, [†]Richard Pestell and [†]Stephen Byers

[†]Lombardi Cancer Center, Georgetown University School of Medicine Washington DC, 20057; [+]Simmons Cancer Center, University of Texas Southwestern, Dallas TX; * Salk Institute for Biological Studies, San Diego, CA

Send Correspondence to:

Stephen W. Byers, PhD, Lombardi Cancer Center, Georgetown University

Research Building E415, 3970 Reservoir Rd. NW

Washington, DC 20057

202-687-1813 (voice)

202-687-7505 (fax)

byerss@georgetown.edu

Running Head: **TNFα regulation of β-catenin signaling**

Key Words: β-catenin, TNF, IKK, cyclin D1, APC.

Abstract

The wnt/β-catenin and TNF/IκB/NF-κB pathways are involved in cell cycle control, differentiation, and inflammation. Both IκB and β-catenin are regulated by phosphorylation at similar consensus serines and targeted for ubiquitination and degradation by the same ubiquitin ligase complex. The IκB kinase complex (IKK) that phosphorylates IκB contains two kinases, IKKα and IKKβ, which are activated in response to cytokines such as TNFα. We now show that TNFα inhibits β-catenin signaling activity independently of the tumor suppressor gene APC. Using a variety of approaches, including dominant negative IKKs, RNAi and IKK knockout cells and animals we show that IKKα and IKKβ but not NFκB mediate the effects of TNFα on β-catenin signaling activity. Although neither TNFα nor constitutively active IKKs reduce total β-catenin protein levels they markedly reduce the level of active dephosphorylated β-catenin in the nucleus by targeting serine and threonine residues in the N-terminal regulatory domain of β-catenin. Levels of β-catenin and the β-catenin target gene cyclin D1 were elevated in the nuclei of epidermal cells in the abnormal skin of IKKα-/- mice. These data point to a role for cytokines and the IKK complex, in the normal regulation of β-catenin signaling activity.

Introduction:

The wnt/β-catenin and TNF/IκB/NFκB pathway regulate a number of processes important in oncogenesis, development and the immune system. These processes include apoptosis, cell cycle control, adhesion and inflammation. In the absence of wnt-signaling, β-catenin is phoshorylated sequentially by casein kinase 1 (CK1) and glycogen synthase kinase 3-β (GSK-3β) (4,44); activation of frizzled receptors by wnt inhibits GSK-3β activity (44). Upon wnt signaling, β-catenin accumulates in the cytoplasm and nucleus where it interacts with Tcf/LEF transcription factor complexes to regulate the expression of genes including cyclin D1 and c-myc (8). Cytokines, such as TNFα stimulate the NFκB signaling pathway, through the activation of many upstream kinases, including GSK-3β and the IκB kinases IKKα and IKKβ (12,13,23,57). In the absence of cytokines, IκB proteins inhibit NFκB transcriptional activity by sequestering it in the cytoplasm (3). Activation of the IKK phosphorylates IκB resulting in its degradation and the release and nuclear translocation of NFκB, which is then able to activates gene transcription (4,52). Both IκB and β-catenin are phosphorylated at similar consensus serines at the N-terminal and targeted for ubiquitination and degradation by the same ubiquitin ligase complex (2,2,27). β-catenin N-terminal phosphorylation also appears to regulate its transactivation properties independently of protein degradation (15). IKKα and IKKβ are components of a 600-900 kDa complex which also includes IKKγ/NEMO which has no catalytic activity but acts as a scaffold protein (31,35,40,56). Several studies have indicated that the wnt and NFκB pathways interact although it is not clear how this occurs (1,28,29). For

example, in PC12 cells, wnt-1 can activate NFκB through inhibition of GSK-3β. In contrast, the GSK-3β-/- mouse is deficient in NFκB transactivation but has no clear wnt signaling phenotype (19). Wnt/β-catenin signaling induces the expression and activity of the ubiquitin ligase complex responsible for the targeting of β-catenin and IκB for ubiquitination resulting in their accelerated degradation and in the case of the IκB pathway, increased NFκB transcriptional activity (26). In a recent study, we showed that the IKK complex, also interacts with and phosphorylates β-catenin and can regulate β-catenin-dependent transcriptional activity (27). Finally, exogenous expression of the active component of NFκB, rel-A can suppress β-catenin signaling (34). Using a variety of approaches, including dominant negative IKKs, RNAi and IKK knockout cells and animals we show that IKKα and IKKβ but not NFκB, APC or GSK-3β mediate the effects of TNFα on β-catenin signaling activity. In contrast, manipulation of β-catenin protein levels or signaling activity does not affect NFκB activation. Activation of the IKK complex does not alter total β-catenin protein levels or localization in APC-negative colon cancer cells but markedly alters the distribution of the activated, de-phosphorylated form of β-catenin. Our data indicate a novel form of regulation of β-catenin that may be important in understanding its role in inflammation, development and in cancer.

Materials and Methods:

Cell culture and Reagents: SW480 cells colon cancer cells were purchased from ATCC and grown in Dulbecco's modified Eagle's medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 5% fetal bovine serum and 2mM L-glutamine. HEK 293 human

embryonic kidney cells were also grown in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum and 2mM L-glutamine. IKKα(-/-) and IKKβ(-/-) mouse embryo fibroblasts (MEF) cell lines were kindly donated by I. Verma and U. Verma. They were grown in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1% penicillin/streptomycin.

Plasmid Constructs and Transfections: SW480 and HEK 293 cells were transfected according to the manufacturer's recommendations using FuGENE 6 Reagent (Roche Diagnostics). IKKα(-/-) and IKKβ(-/-) mouse embryonic fibroblast cells were transfected using Lipofectamine Plus (Invitrogen, CA) according to the manufacturer's recommendations. F. Mercurio kindly provided the IKKα (S176/180E, also known as constitutively active, CAIKKα and S176/180A, also known as dominant negative, DNIKKα) and IKKβ (S177/181E, also known as constitutively active, CAIKKβ and S177/181A, also known as dominant negative, DNIKKβ) and IKKα (K44M) and IKKβ (K44M) mutants (36,54). B. Vogelstein kindly provided the APC plasmid, W. Li provided the dominant negative mutants to PI3 kinase and AKT, C. Duckett provided K63W TAK1 and TAB1, and the siamois reporter plasmid was provided by Kimelman D.

Deletion constructs of β-catenin were made via PCR using primers of the following:

ΔN: forward primer 5'- CGGGATCCATGCGTGCAATCCCTGAACTG -3' reverse primer 5'- GCTCTAGATCATTACAGGTCAGTATCAAACCAGGCC-3' ΔC: forward

primer 5'- CGGGATCCATGGCTACTCAAGCTGATTTG -3' reverse primer 5'-GGCTCTAGAGCCTTATGCGTAGTCCGGCACGTCGTACGGGTAAGAGCTGGTC AGCTC -3' The inserts were digested using restriction enzymes, BamH1 and Xba1. They were ligated into pcDNA3 vector (Invitrogen), transformed and selected with ampicillin.

Site Directed Mutagenesis: Mutations were performed using Pfu proofreading polymerase and QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene) with modifications made to the PCR cycling termperatures. The following primers were used:

T41A: 5'- CTGGTGCCACTGCCACAGCTCCTTCTCTG -3'.

S45A: 5'- CCACAGCTCCTGCTCTGAGTGGTAAAGGC -3'

S33A, S37A: 5'- CAGTCTTACCTGGACGCTGGAATCCATGCT-3' S33A, S37A mutant was made using the S37A template in pcDNA3 vector (Invitrogen)

S33A, S37A, T41A, S45A: 5'- TACCTGGACGCTGGAATCCATGCTGGTGCC -3' S33A, S37A, S45A, T41A mutant was made using the S33A, S41A, S45A mutant template.

Tcf/LEF Reporter Assay: Cells were plated at 100,000 cells/well and experiments were performed in 12 well cell culture plates. 24 hours after plating, the cells were transfected with the indicated DNA and one of the reporters; TOPFlash (0.1ug), FOPFlash (0.1ug) or NF-kB luciferase (0.1ug). Dose responses of dn IKKα and dn IKKβ ranged from 0.05-1.0 ug. 0.2ug of β-catenin and their point mutants were used to transfect MEFs. Renilla luciferase reporter (1ng) was used as an internal control to correct for transfection efficiency. All transfections were done in triplicate and repeated at least 3 times with the

Tcf/LEF reporter activity measured in lumens after 16 hours of TNFα treatment or 24 hours after transfections using Dual Luciferase Reporter Assay System kit (Promega). Dose responses were also performed and optimal doses were chosen for these experiments.

RNA oligonucleotides: siRNAs with two thymidine residues (dTdT) at the 3' end of the sequence were designed for

IKKα (sense 5'- AAGCAGGCTCTTTCAGGGACACCTGTCTC -3'),

IKKβ (sense 5'- AAGTCGACGGTCACTGTGTACCCTGTCTC -3'),

p65 (sense 5'- AAGCCCTATCCCTTTACGTCACCTGTCTC -3'),

non-specific RNA oligonucleotides (sense 5'- AAGAGGGGGGTTCCATTAAGCCCTGTCTC -3') genes along with their corresponding antisense RNA oligonucleotides as described. (*) These RNAs were dissolved in RNase free water as 100μM solutions. RNA oligonucloetides targeting cadherin

Transfections of RNA oligonucleotides: Approximately, 1 X 10^6 cells were plated per 12-well plate in containing 5% FBS to give 40-50% confluency and transfection of the RNA oligonucleotides was performed using Oligofectamine (Invitrogen, Carlsbad, CA) to result in a final RNA concentration of 25nM for IKKα and 100nM for IKKβ and p65. The cells were transfected consecutively with the oligonucleotides for 2 days and they were harvested 48 hours post-transfection with TOPFlash and NFκB reporters. They were treated with TNFα 24 hours post-transfection.

Western Blotting: Cells were grown to confluence in 100mm dishes (48 hours after transfection) and washed twice with phosphate buffered saline (PBS). They were lysed for 10 minutes on ice in NP-40 lysis buffer (1% NP-40 and protease inhibitors including 1mM sodium vanadate, 50mM sodium fluoride and Boerhringer Mannheim complete mini EDTA-free protease inhibitor cocktail tablet). Lysates were centrifuged at 14,000 rpm at 4°C. For pure cytoplasmic lysates, cells were washed with PBS as described and lysed for 10 minutes on ice in Swell Buffer (10mM Tris pH7.5, 0.2mM magnesium chloride). Cells were scraped and dounced, after which, inhibitor cocktail (1.25M sucrose, 250mM sodium fluoride, 5mM EDTA, 5mM sodium vanadate and Boerhringer Mannheim complete mini EDTA-free protease inhibitor cocktail tablet) was added. Lysates were centrifuged at 28,800 rpm at 4°C for 1 hour. The supernatant was mixed with ethanol and left overnight at -20°C. This was then centrifuged at 28,800 rpm at 4°C for 30 minutes. The precipitate was dissolved in sample buffer (2% sodium dodecyl sulphate, 60mM Tris, pH6.8, 10% glycerol). Protein content was measured by BCA protein assay (Pierce Laboratories). 3-8% tris-acetate NuPage gels (Invitrogen Corporation) were used for SDS-PAGE. Monoclonal antibody to β-catenin was obtained from Transduction Laboratories. Monoclonal antibody against HA tag (Boerhinger Mannheim), monoclonal FLAG antibody (Eastman Kodak) and monoclonal antibodies against IKKα and IKKβ (Upstate Biotechnology) were also used in Western blot analysis and immunocytochemistry. De-phosphorylated β-catenin antibody was kindly donated by Hans Clevers.(51) The blots were developed using ECL chemiluminescent detection (Amersham Pharmacia Biotech). Some blots were stripped (62.5mM Tris pH 7.5, 2%

SDS, 1.7% β-mercaptoethanol) for 25 minutes at 50°C, re-blocked with 5% non-fat milk and re-probed with new primary and secondary antibody according to manufacturer's recommendations.

Immunocytochemistry: Cells were grown to confluence in 175 cm^2 flasks and trypsinized using trypsin (Invitrogen Corporation) where 150,000 cells per well were plated on 18mm cover slips in 12 well plates. The cells were transfected as described above with 1ug of HA-tagged CAIKKα or Flag-tagged CAIKKβ. After 48 hours, they were washed twice with phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde at room temperature for 30 minutes and permeabilized in 0.2% TritonX-100 at room temperature for 5 minutes. With TNFα treatment, the cells were treated with it for 16 hours and similar procedures were carried out. Purified rabbit HA antibody (Zymed Laboratories) or Flag antibody was used (Sigma-Aldrich) and normal IgG fluorescein or Texas Red® dye-conjugated (Kirkegaard and Perry Laboratories) were used as secondary antibodies for staining. All primary antibodies were incubated overnight at 4°C and all secondary antibodies were used at 1:500 dilution at room temperature unless otherwise noted above. Imaging was done using a Nikon E600 Flourescence Microscope System with FITC or TRITC filter.

Immunohistochemistry: YinLing Hu and Michael Karin kindly provided the IKKα knockout and wild-type mouse paraffin blocks (20). Paraffin sections were dewaxed in xylene and rehydrated sequentially with 100, 95 and 50% ethanol in water. Sections were boiled in a solution of 10mM sodium citrate, pH 6.0 and microwaved on high power for

3x3 minute cycles, with 1 minute between cycles. They were allowed to cool for 30 minutes, rinsed 3 times in PBS and incubated in 0.3% peroxide solution at 4°C for 30 minutes. They were rinsed in PBS and air dried, after which the samples were circled with a PAP pen. The following antibodies were used: mouse IgG (Zymed Laboratories) and monoclonal β-catenin (Transduction Laboratories). Sections were incubated in horse anti-mouse biotinylated secondary antibody (Vector Laboratories) at room temperature for 60 minutes. The AEC Substrate Kit (Zymed Laboratories) was used to detect staining and some sections were lightly counterstained with hematoxylin. Cyclin D1 immunocytochemistry was also carried out as described earlier.(33)

Results:

TNFα affects β-catenin signaling through the IKK complex and independently of APC: In previous studies, we reported that treatment of β-catenin-transfected HeLa cells with TNFα activated the IKK complex and increased the phosphorylation of β-catenin (27). To further understand how TNFα regulates β-catenin, we studied its effect on β-catenin signaling in SW480 colon cancer cells. These cells have a truncated APC gene and thus have high endogenous β-catenin protein levels and signaling. To measure β-catenin signaling, we used a luciferase promoter containing Tcf/LEF response elements (Topflash) (49). SW480 cells express the TNFα receptor and treatment with the ligand results in apoptosis after 48h (17,22). Treatment of SW480 cells with 20ng/ml TNFα for 16 hours significantly increased NFκB reporter activity and decreased β-catenin reporter activity (**Fig. 1A**). Similar results were obtained in 293 cells, which have lower (but still

detectable) endogenous levels of β-catenin signaling and express wild-type APC (**Fig. 1B).** TNFα also inhibited β-catenin/TCF signaling in 293 cells transfected with β-catenin (not shown) as well as the activity of the β-catenin/TCF sensitive siamois reporter in a similar manner **(Fig. 1C)**. The siamois reporter contains Tcf/LEF sites as well several other potential transcription factor binding sites (6) However, the effects of TNFα on siamois promoter activity are mediated by the interaction of β-catenin with Tcf/LEF sites since TNFα had little effect on the activity of a siamois promoter in which the TCF sites were mutated. No significant changes in the activity of a renilla-luciferase control reporter occurred and we did not observe any cell death after the 16 h treatment period. Thus our data show that the effect of TNFα on β-catenin signaling is independent of APC (it works both in the absence (SW480 cells) and presence (293 cells) of APC) but does depend on TCF binding elements.

TNFα exerts its effects on NFκB by activating the IKK complex (11,58). NFκB-inducing kinase (NIK) can also activate the IKK complex but does so independently of TNFα. We next wanted to test the hypothesis that activation of IKK, either by TNFα or NIK could regulate β-catenin signaling. Like TNFα, expression of NIK decreased β-catenin signaling and increased NFκB activity (**Fig. 1D**). To further relate the activation of the IKK complex with β-catenin signaling, we transfected SW480 cells with constitutively active IKK mutants and Topflash. The constitutively active mutants (CAIKKα and CAIKKβ) were generated by changing serines residues located within the highly conserved activation loops at serines 176 and 180 in IKKα and 177 and 181 in IKKβ to

glutamate (SS to EE) (36). This mimics the phosphorylation induced conformational change to result in kinase activation. β-catenin signaling decreased by 80-90% upon transfection of either CAIKKα or CAIKKβ. **(Fig. 1E)** The effects of CAIKKα and CAIKKβ on β-catenin and NFκB activity were further augmented by treatment of cells with TNFα **(Fig. 1E)**. Kinase inactive mutants of IKKα and IKKβ did not inhibit β-catenin signaling activity **(Fig. 1F)**. Similar results were obtained using the cyclin D1 promoter **(Fig. 1G)**. Because NFκB positively regulates cyclin D1 promoter activity, these data indicates that in APC-mutant colon cancer cells, TNFα regulation of the –163 cyclin D1 promoter is predominantly regulated by the β-catenin/TCF and not the NFkB site (46). Consistent with this, the activity of a cyclin D1 promoter construct with a mutated TCF site was unaffected by CAIKKs.

The IKK complex mediates the effects of TNFα but not APC on β-catenin signaling: In order to identify the relative contribution of IKKα and IKKβ in the TNFα-induced down-regulation of β-catenin reporter activity, we transfected SW480 cells with dominant negative IKKα (S176/180A) or dominant negative IKKβ (S177/181A) **(Fig. 2A)**. The alanine substitutions for serine residues in the activation loops of these kinases inhibit kinase activation by upstream mediators. Both dominant negative mutants effectively blocked the down-regulation of Topflash and induction of NFκB reporter by TNFα. Similar results were observed with the siamois reporter **(Fig. 1C)**. Like TNFα, expression of wild-type APC in APC-mutant SW480 cells also inhibits β-catenin signaling. Although β-catenin signaling was reduced dramatically by APC, NFκB

reporter activity was completely unaffected and DNIKKs did not affect the ability of APC to inhibit β-catenin signaling activity **(Fig. 2B)**. In addition, APC did not potentiate or inhibit the ability of TNFα to regulate NFκB activity in SW480 cells.

We also used RNAi to inhibit IKKα and IKKβ expression (**Fig. 2C-D**). Repression of β-catenin signaling by TNFα was markedly inhibited in the presence of either IKKα or IKKβ RNAi (Fig. X). Even though our results are related to non-specific RNAi we were concerned that the large amount of IKKβ RNAi that was used in these experiments may have adverse non-specific effects on the cell. To confirm the role of IKKα and IKKβ in TNFα regulation of β-catenin signaling, we used mouse embryonic fibroblasts (MEFs), which were disrupted in IKKα or IKKβ and performed similar experiments (21,32). When either IKKα(-/-) or IKKβ(-/-) or MEFs were used, TNFα was unable to down-regulate β-catenin signaling activity (**Fig.2E**). NFκB signaling in response to TNFα was also significantly inhibited in the IKKα-/- and IKKβ-/- MEFs. Transient transfection of wild type IKKα or IKKβ into the knockout cells restored TNFα responsiveness (**Fig.2F-G**). Taken together these data show unequivocally that IKKα and IKKβ are involved in TNFα regulation of β-catenin signaling activity. Although NIK can activate the IKK complex and decrease β-catenin signaling (**Fig. 1D**), dominant negative NIK could not reverse the effects of TNFα on Topflash or NFκB activity, consistent with previous data showing that NIK is not required for TNFα activation of IKK (41)(**Fig. 2E).** Although it does not activate NIK, TNFα can activate a number of kinases other than IKK, including, PI3 kinase, Akt, and importantly, GSK-3β (7,19,38,45). We next tested the role of these

kinases by transfecting dominant negative PI3 kinase, Akt, and by treating cells with LiCl to inhibit GSK-3β. None of these manipulations affected the ability of TNFα to inhibit β-catenin signaling further pointing to the important role of the IKK complex in mediating the effects of TNFα on β-catenin signaling (**Fig. 2G**).

TNFα repression of β-catenin activity is independent of NFκB signaling activity: We showed previously that IKK effects on β-catenin signaling in β-catenin transfected COS cells were unaffected by inhibition of NFκB activation by mutated IκB (25). We also found that mutated IκB did not influence the effects of CAIKK mutants on β-catenin signaling in SW480 cells even though it completely inhibited the activation of NFκB (not shown). This indicates that inhibition of NFκB activation downstream of IKK does not affect β-catenin signaling. However, there is some evidence that exogenously over-expressed NFκB may play a role in the regulation of β-catenin signaling activity by suppressing β-catenin dependent transcription (10,34). We wanted to confirm if TNFα mediated repression of β-catenin signaling is indeed independent of TNFα activation of NFκB. Of the five NFκB subunits in mammalian cells, RelA (p65), RelB and c-Rel contain transactivation domains in the C-terminus and require no additional processing to their active forms (53) We found that SW480 cells and MEFs express p65 and used siRNA to silence it. Our western analysis confirmed that p65 was silenced following treatment (**Fig. 3A**) and that loss of p65 prevented the activation of the NFκB reporter by TNFα (**Fig. 3B**). However, even in the absence of p65 protein TNFα continued to repress

β-catenin signaling activity (**Fig.3C**). This data strongly suggests that TNFα regulates β-catenin signaling activity independently of NFκB.

TNFα and activated IKK regulate the localization of activated, dephosphorylated β-catenin: Earlier studies showed that TNFα and CAIKK mutants resulted in phosphorylation of exogenously expressed β-catenin (27). We showed several years ago that phosphorylation of certain serine residues in the N-terminal of β-catenin can regulate its stability by targeting it for ubiquitination (27) Although a putative IKK consensus sequence is present in the N-terminal region of β-catenin and is a likely target for IKK, mapping studies indicated that several other regions of β-catenin can serve as substrates for IKK (27). It is also possible that IKK may affect β-catenin mediated trans-activation by phosphorylating another component of the transcriptional machinery. If TNFα or activation of IKK regulates β-catenin signaling by targeting it for ubiquitination and protein degradation, one would expect that like APC, axin or activated GSK-3β they would affect β-catenin protein levels (24,37). However, treatment with TNFα did not alter cytoplasmic protein levels of total β-catenin in SW480 cells as measured by western blotting with an antibody directed at the C-terminal **(Fig. 4A).** In 293 cells, which have much less cytoplasmic β-catenin than SW480 cells treatment with TNFα actually appeared to <u>increase</u> β-catenin protein levels **(Fig. 4B)**. Immunocytochemical studies showed that treatment with TNFα had no obvious effect on β-catenin protein localization (results not shown). Similarly, double-labeling studies of β-catenin in SW480 cells transiently transfected with CAIKKs revealed no significant alteration in β-catenin levels

or localization in the cells expressing IKKs (**Fig. 5A-C**). This is in marked contrast to SW480 cells transfected with APC or axin/conductin in which addition of these components of the β-catenin degradation pathway results in a marked loss of nuclear and cytoplasmic β-catenin concomitant with changes in Topflash activity (16,18). Some studies have shown that phosphorylation of β-catenin on serines 33, 37, 45 and threonine 41 regulates its cellular localization and transcriptional activation functions, in addition to or instead of regulating protein degradation (27,43,44,50,51). In addition, IKKs directly affect the transcriptional activity of NFκB as well as targeting IκB for degradation (39,55). Recently, reagents that specifically recognize phosphorylated (inactive) or dephosphorylated (active) forms of β-catenin have been developed (47). In untreated SW480 cells activated β-catenin was localized to the nucleus and occasionally at cell-cell contact sites. Remarkably and in contrast to total β-catenin, activated β-catenin was almost always absent from the nuclei of cells transiently transfected with CAIKKα or β (**Fig. 5D-F**). No change in the localization of activated β-catenin was observed in cells expressing DNIKKs (not shown). These data are quantitated in figure 5G-J. Similar results were obtained in SW480 cells treated with TNFα, in which activated β-catenin was significantly reduced in the nucleus (**Fig. 6A-B**). These data are quantitated in figure 6G. Note that this quantitation does not take into account cells with modestly reduced but still detectable nuclear localization of activated β-catenin after treatment with TNFα and therefore represent an underestimate of the effects of TNFα. These data indicate that the effects of TNFα on β-catenin signaling are not associated with increased degradation of total β-catenin but do involve changes in the localization/level of activated β-catenin.

TNFα trans-repression of β-catenin activity involves phosphorylation of N-terminal serine and threonine residues:

We wanted to investigate if phosphorylation sites within the N-terminus might be involved in β-catenin regulation by TNFα. SW480 cells were stained with antibodies that recognize β-catenin only when it is phosphorylated on serines 41 and 45 (21). Little staining was observed in control cells but a dramatic increase in nuclear staining was observed 5-15 minutes following treatment with TNFα (**Fig. 6C-D**). Treatment of SW480 cells with TNFα also resulted in a change in staining using an antibody specific for β-catenin when it is phosphorylated on serines 33, 37 and threonine 41. This staining pattern was cytoplasmic/membrane rather than nuclear. (**Fig 6E-F**) Similar results were observed in HEK293 cells (not shown). These data indicate that one or more of the N-terminal serine residues are important for TNFα to trans-repress β-catenin activity. To formally test this hypothesis we transfected MEFs with β-catenin, ΔNβ-catenin or β-catenin mutated either, on residues 33,37,41,45 or on serine 45 alone. Expression of β-catenin in wild-type MEFs increased β-catenin signaling and treatment of transfected cells with TNFα decreased this exogenous signaling activity (**Fig 7A**). In contrast, wild type MEFs transfected with ΔNβ-catenin, β-catenin mutated on 33,37,41,45 or on serine 45 alone were resistant to repression by TNFα. Interestingly, TNFα significantly increased the already elevated activity of β-catenin mutated on 33,37,41,45 or on serine 45. As expected IKK-/- MEFs transfected with either wt β-catenin or the various β-catenin mutants were insensitive to repression by TNFα (**Fig. 7B-C**). Taken together these data suggest that serine 45 is a key residue in the regulation of β-catenin repression

by TNFα and IKK. To specifically test this we used another colon cancer cell line, HCT116, which has normal APC but expresses one allele of β-catenin mutated on serine 45 and one normal allele. We used parental HCT116 cells and HCT116 cells in which either the normal or the mutated β-catenin allele has been removed by somatic cell knockout (25). Remarkably, cells in which the normal allele had been removed (only expressing mutant β-catenin) were completely resistant to the effects of TNFα whereas cells in which the mutant allele was deleted (only expressing normal β-catenin) were highly sensitive and the parental cell line only moderately sensitive (**Fig. 7D**).

β-catenin and cyclin D1 are localized in the nuclei of epidermal cells in the neonatal IKKα -/- mouse

Our results indicate a role for the IKK complex in the regulation of β-catenin localization and signaling. A number of studies indicate that IKKα does not play a dominant role in the regulation of NF-kB signaling (9,30,32). Although the IKKα(-/-) mouse does not have a marked NFκB phenotype, it does exhibit a thickening of the epidermis and has a defect in keratinocyte differentiation (20,48),(30). Moreover, Hu et. al. had shown that the role of IKKα in keratinocyte differentiation is not exerted through IKK activation of NF-kB (21). Because elevated β-catenin signaling is associated with hyper-proliferation of epidermal basal cells, and the wnt/β-catenin/TCF pathway is implicated in epithelial stem cell proliferation and differentiation, we investigated β-catenin localization in the epidermis of IKKα (-/-) mice. Unfortunately, we were unable to use the antibody to activated β-catenin on paraffin sections but were able to use an antibody directed against

the C-terminal of β-catenin. Membrane β-catenin staining was observed in both wild-type and IKKα (-/-) epidermis but a marked increase in the number of cells with nuclear β-catenin occurred throughout the epidermis and particularly in the basal region of IKKα (-/-) mice (**Fig. 9A**). Very few cells in the normal epidermis exhibited nuclear β-catenin staining at this stage in development. Nuclear β-catenin staining also occurs in human epidermal keratinocytes expressing N-terminal deleted form of β-catenin but not in normal keratinocytes or in normal human skin (59). Localization of β-catenin in the nucleus suggests an increase in β-catenin/TCF signaling. Thus we localized the β-catenin target gene cyclin D1. Like β-catenin, cyclin D1 was markedly increased in the basal regions of the IKKα (-/-) mouse epidermis. (**Fig. 9B**)

Discussion:

Cells produce a wide array of cytokines, which are important for the generation of inflammatory and immune responses. TNFα and other members of the TNF family also play a role in a variety of oncogenic and developmental processes and there are several indications of interaction between TNF/IKK/NFκB and wnt/GSK-3/β-catenin pathways. For example, the TNFα-activated kinases IKKα and IKKβ can interact with and phosphorylate β-catenin (27). GSK-3β and NFκB also appear to exert direct regulation over both pathways (19). In the present study we show that IKKα and IKKβ but not NFκB mediate the effects of TNFα on β-catenin signaling activity. In addition β-catenin and the β-catenin target gene cyclin D1 accumulated in the nuclei of epidermal cells in the abnormal skin of IKKα (-/-) mice. These data, together with other published studies point to a role for cytokines and the IKK complex in the normal regulation of β-catenin signaling activity.

TNFα/IKK-mediated phosphorylation of β-catenin affects its signaling activity but does not result in a change of total β-catenin levels:

In SW480 cells neither TNFα nor constitutively active IKKs affect total β-catenin protein levels or localization even though they rapidly alter β-catenin N-terminal phosphorylation and signaling activity. This is in contrast to the effects of APC, axin or activated GSK-3, which all result in the degradation of total β-catenin, concomitant with increased N-terminal phosphorylation and decreased signaling activity. These data raise the possibility

that phosphorylation of the same N-terminal residues targets β-catenin for degradation in the context of APC/axin/GSK-3 but not in the context of TNFα/IKK. Although this seems contradictory it is clear that phosphorylation of β-catenin on serines 33, 37, 45 and threonine 41 can regulate its cellular localization and transcriptional activation functions, in addition to regulating protein degradation (27,42,44,50,51). In Xenopus oocytes β-catenin N-terminal phosphorylation regulates its transactivation properties completely independently of protein degradation (14). In addition, IKKs that have translocated to the nucleus following cytokine treatment directly affect the transcriptional activity of NFκB as well as targeting IκB for degradation (39,55). It is also possible that TNFα/IKK targets a small pool of transcriptionally active β-catenin for degradation. Exogenous expression of E-cadherin can inhibit β-catenin/TCF signaling in SW480 cells in the absence of discernible changes in cytoplasmic or nuclear β-catenin (Gottardi, 2001 2349 /id}. These data led the authors to conclude that most of the β-catenin in SW480 cells is refractory to cadherin and TCF binding and that a small pool of transcriptionally active β-catenin can be modulated by E-cadherin. In other experiments we show that mechanical strain also affects β-catenin signaling activity and nuclear "activated" β-catenin without altering total β-catenin protein levels (Avvisato, Byers et al submitted for publication).

Our data show that β-catenin phosphorylated on residues 45 and 41 appears in the nucleus as early as 5 minutes following TNFα treatment (**Fig X**). Although no clear change in the staining of activated de-phosphorylated β-catenin occurs at these early time points, the antibody that recognizes activated β-catenin is actually directed at β-catenin

that is de-phosphorylated on residues 37 and 41 and will still recognize β-catenin phosphorylated on residue 45 (51). A clear loss of activated β-catenin staining from the nucleus is detected 16 hours after TNFα treatment. This means that this pool of β-catenin is either phosphorylated on residues 37 and 41 or that it has been degraded and no longer exists. At this time no nuclear staining with either phospho-β-catenin antibody is observed indicating that the latter is more likely. Consequently, one plausible interpretation of our data is that phosphorylation of a small pool of nuclear activated β-catenin by activated IKK and perhaps other kinases results in its degradation and/or alters its co-activator properties. β-catenin degradation could take place in the nucleus or following export into the cytoplasm. Consistent with this interpretation, one study has shown that β-catenin associated with the nuclear matrix in melanoma cells is a target for proteosomal degradation independently of APC (5).

TNFα repression of β-catenin activity is independent of NFκB signaling activity:

Two studies have shown that exogenously over-expressed NFκB may play a role in the regulation of β-catenin signaling activity by directly interacting with it (10,34). We wanted to confirm if TNFα mediated repression of β-catenin signaling is indeed independent of TNFα activation of NFκB. Using RNAi to deplete p65 we showed that even in the absence of p65 protein TNFα continued to repress β-catenin signaling activity. This data demonstrates that TNFα regulates β-catenin signaling activity independently of NFκB. Consistent with this we showed previously that IKK effects on β-catenin signaling in β-catenin transfected COS cells were unaffected by inhibition of

NFκB activation by mutated IκB (25). This indicates that NFκB activation downstream of IKK does not mediate TNFα repression of β-catenin signaling. However, these data do not exclude a role for direct β-catenin/NFκB interactions in the regulation of promoters containing both TCF/LEF and NFκB sites. Indeed in other studies we show that the β-catenin/TCF sensitive FGF-BP and cyclin D1 promoters are co-ordinately regulated by both NFκB and β-catenin/TCF sites (and unpublished results).

Conclusion:

Our immunohistochemical analysis indicate that β-catenin and its target gene, cyclin D1 are upregulated in the nuclei of IKKα (-/-) epidermal mice cells. We have found that TNFα represses β-catenin signaling through the IKK complex and this occurs independently of APC. Through RNA silencing technique, we have also determined that this repression is independent of NFκB signaling activity. We have found that TNFα and activated IKK regulate the localization of β-catenin that is not phosphorylated on Ser37 and Thr41. This pool of 'active' β-catenin is significantly reduced in the nucleus after treatment with TNFα. We have also determined that TNFα regulates the phosphorylation of N-terminal serine and threonine residues. It is important to remember that β-catenin is overexpressed in many cancers including the skin. It is interesting to observe that a pleotrophic cytokine such as TNFα, which plays a major role in the pathogenesis of many autoimmune and chronic inflammatory diseases is able to regulate β-catenin activity. Our findings have brought about further insight on another way by which β-catenin may be regulated besides the well-known wnt pathway.

Acknowledgements

We thank Drs. Michael Karin and YinLing Hu for the paraffin blocks from the IKK-/- animals, Drs. Inda Verma, Mark Schmidt and Udit Verma for the IKK (-/-) cells, Dr. Todd Waldman for this HCT 116 cells and Dr. Richard Pestell for the cyclin D1 promoters. This work was supported by a grant from the U.S. Army Medical Research and Materiel Command for Breast Cancer.

Legend to Figures:

Figure 1A: SW480 cells were treated with 20ng/ml of TNFα for 16 hrs and assayed for the luciferase act activities of different reporters. TNFα significantly increased the activity of NFκB reporter, and decreased the activity of TOPFLASH reporter. TNFα had no significant effect on FOPFLASH.

Figure 1B: 293 cells were similarly treated with 20ng/ml of TNFα for 16 h and assayed for luciferase activities of different reporters. Similar results as Fig.1 were obtained, indicating that the effects of TNFα on β-catenin signaling are independent of APC.

Figure 1C: SW480 cells treated with 20ng/ml of TNFα and increasing amounts of dominant negative IKK (SS/AA) or IKK (SS/AA). a) 150ng of reporter Cyclin D1-163 was used as a measure of TOPFLASH activity. TNFα and the mutants did not have any significant effect on its corresponding mutant, LEF. b) 150ng reporter Siamois was used as a measure of TOPFLASH activity.

Figure 1D: SW480 cells were transfected with NFκB inducing kinase (NIK) together with its reporters, TOPFLASH or NFκB. NIK repressed TOPFLASH activity and augemented NFκB reporter activity.

Figure 1E: CAIKKα and CAIKKβ decreased β-catenin signaling in SW480 cells. The same effect was seen when endogenous IKKs were activated by treatment with TNFα. NFκB signaling was significantly induced in cells treated with TNFα and those transfected with CAIKKα and β.

Figure 1F: SW480 cells transfected with kinase dead IKK mutants which are unable to repress β–catenin signaling activity.

Figure 1G: SW480 cells transfected with constitutively active IKK mutants. Both mutants further decreased b-catenin signaling activity with cyclin D1 promoter. Mutant DLEF cyclin D1 had little change compared to the control as expected.

Figure 2A: TNFα significantly reduced β-catenin signaling in SW480 cells. This was reversed in a dose-dependent pattern when they were transfected with IKK (SS/AA) or IKK (SS/AA). Both dominant negative mutants blocked TNFα-induced increase in NFκB signaling.

Figure 2B: APC decreases β-catenin signaling by 80% in SW480 cells and this effect could not be reversed by DNIKK or DNIKK. Both forms of dominant negative IKKs block TNFα-induced increase in NFκB signaling and this effect is independent of APC.

Figure 2C: SW480 cells with 25nM non-specific RNAi (NSRNAi) or RNAi against IKKα treated with or without TNFα. In the presence of IKKα RNA, the cells are not responsive to TNFα and β-catenin signaling activity is not repressed.

Figure 2D: SW480 cells with 100nM non-specific RNAi (NSRNAi) or RNAi against IKKβ treated with or without TNFα. In the presence of IKKβ RNA, the cells are not responsive to TNFα and β-catenin signaling activity is not repressed.

Figure 2E: Wild Type MEF cells (a), IKKα-/-(b), IKKβ-/-(c) cells were transfected with TOPFLASH reporter and renilla and treated with 20ng/ml of TNFα. The knockout cell lines were resistant to TNFα and no down-regulation of β-catenin signaling was seen, contrary the wild type cell line as expected. NFκB signaling is still augmented with the treatment of TNFα in all cell lines, confirming that the increase is independent of the decrease in β-catenin signaling.

Figure 2F: IKKα-/- MEF cells with wild type IKKα restored, showed a sensitivity to TNFα (20ng/ml) treatment with a decrease in β–catenin signaling activity.

Figure 2G: IKKβ-/- MEF cells with wild type IKKβ restored, showed a sensitivity to TNFα (20ng/ml) treatment with a decrease in β-catenin signaling activity.

Figure 2H: Kinase dead NIK mutant is unable to reverse TNFα repression of β-catenin signaling activity. Other mutants such as AKT, PI3kinase and TAK1 are also unable to reverse its effects.

Figure 3A: Western analysis of wild type MEFs were transfected with p65 RNA oligonucleotides show significant reduction of p65 protein compared to non-specific RNAoligonucleotides (NSRNAi) and mock transfections.

Figure 3B: Topflash reporter assay. TNFα is still able to down-regulate β-catenin signaling activity with the presence of p65 RNA.

Figure 3C: NFκB reporter. Treatment with p65 RNA significantly reduces NFκB signaling activity compared to control.

Figure 4A: Top panel: Cytoplasmic fraction obtained from SW480 cells were treated with 20ng/ml TNFα harvested after 16 hrs. They were probed with β-catenin antibody that recognizes C-terminal. No significant changes were observed in treated (+) lanes compared to control (-). Lower panel: Loading was done using α-tubulin.

Figure 4B: Cytoplasmic fraction obtained from 293 cells treated with 20ng/ml TNFα over a similar period. An increase in β-catenin protein levels are observed after treatment with the cytokine. Loading was done with α-tubulin.

Figure 5: Immunocytochemistry. SW480 cells transfected with CAIKKα or CAIKKβ mutants had no change in β-catenin staining pattern (x40 mag):

Figure 5A: Cells were stained for flag tagged IKK mutants (flourescein) showed strong cytoplasmic staining with little nuclear staining. (see arrow)

Figure 5B: Cells were stained for β-catenin (Texas red) showed predominantly nuclear with some cytoplasmic staining. (see arrow)

Figure 5C: Combined image of panel a and panel b.

Figure 5D: Staining for de-phosphorylated β-catenin (Flourescein)

Figure 5E: Staining for transfected CAIKK mutants (Texas Red)

Figure 5F: Combined image of D) and E). SW480 cells transfected with CAIKK mutants had little or no nuclear staining (see arrow) when probed with de-phosphorylated form of β-catenin. Compare this to untransfected cells in panel A, which show a strong nuclear stain pattern.

Figure G-H: Quantitation of the cells with and without nuclear staining after transfection with CAIKKα (G) CAIKKβ. 10 fields at x40 mag were taken and average calculated. There is a significant decrease in number of cells with nuclear staining after transfected with CAIKK mutants. A reversal was seen when dominant negative IKK mutants were transfected.

Figure 6: Immunocytochemistry.

A-B: SW480 cells were treated with 20ng/ml of TNFα for 16 hrs and stained for de-phosphorylated β-catenin. At similar exposure times, treatment with TNFα resulted in less intense nuclear staining when compared to control (A).

C-D:SW480 control cells stained with antibody that recognizes β-catenin phosphorylated at Thr41/Ser45. Cells with and without nuclear staining before (C) and after treatment with TNFα (D). Only cells treated with TNFα show a dramatic increase in nuclear staining at 30 min.

E-F: SW480 cells treated with 20ng/ml of TNFα for 30min. SW480 control cells (F) stained with antibody that that recognizes β-catenin phosphorylated at Ser33, Ser37, Thr41 Cells treated with TNFα show an increased staining in the cytoplasm and membrane after 30 min.

Figure 7A: Parental and knockout MEF cells were transfected with wild type β-catenin (WT), S45A mutant or S33,37,41,45 mutant and treated with 20ng/ml TNFα. Mutant β-catenin was insensitive to the effects of TNFα.

Figure 7B-C: Knockout IKK cells were insensitive to TNFα, since β-catenin signaling activity was not significantly repressed.

Figure 7D: Cell line with mutant S45 allele present only, are insensitive to the effects of TNFα compared to parental cells and when the wild type Ser45 allele is present alone.

Figure 8: Both wildtype (WT) (A) and IKKα-/- (B) embryos were stained with mouse IgG and hematoxylin. No background staining was observed with the IgG control antibody. Note the thickened epidermis of the IKKα (-/-) embryo. Because hematoxylin

masked the nuclear β-catenin staining, the embryos were stained with β-catenin alone. Membrane staining was observed in both WT (C) and IKKα (-/-) (D). However, an increased number of epidermal cells with nuclear β–catenin (arrows) was observed in the IKKα (-/-) mouse. Cyclin D1 expression was also increased in the basal aspects of the epidermis of the IKKα (-/-) mouse. (E-H) Cyclin D1 staining was performed in Richard Pestell's laboratory.

Reference List

1. **Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler**. 1997. β-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. **16**:3797-3804.

2. **Albanese, C., K. Wu, M. D'Amico, C. Jarrett, D. Joyce, J. Hughes, J. Hulit, T. Sakamaki, M. Fu, A. Ben Ze'ev, J. F. Bromberg, C. Lamberti, U. Verma, R. B. Gaynor, S. W. Byers, and R. G. Pestell**. 2003. IKKalpha Regulates Mitogenic Signaling through Transcriptional Induction of Cyclin D1 via Tcf. Mol.Biol Cell **14**:585-599.

3. **Amit, S. and Y. Ben Neriah**. 2003. NF-kappaB activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach. Semin.Cancer Biol **13**:15-28.

4. **Amit, S., A. Hatzubai, Y. Birman, J. S. Andersen, E. Ben Shushan, M. Mann, Y. Ben Neriah, and I. Alkalay**. 2002. Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev. **16**:1066-1076.

5. **Bonvini, P., S. G. Hwang, M. El Gamil, P. Robbins, J. S. Kim, J. Trepel, and L. Neckers**. 2000. Nuclear beta-catenin displays GSK-3beta- and APC-independent proteasome sensitivity in melanoma cells. Biochim.Biophys.Acta **1495**:308-318.

6. Brannon, M., M. Gomperts, L. Sumoy, R. T. Moon, and D. Kimelman. A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes Dev. 11(18), 2359-2370. 1997.
Ref Type: Journal (Full)

7. **Burow, M. E., C. B. Weldon, L. I. Melnik, B. N. Duong, B. M. Collins-Burow, B. S. Beckman, and J. A. McLachlan**. 2000. PI3-K/AKT regulation of NF-

kappaB signaling events in suppression of TNF-induced apoptosis. Biochem.Biophys.Res Commun. **271**:342-345.

8. **Chen, S. Q., D. C. Guttridge, Z. B. You, Z. C. Zhang, A. Fribley, M. W. Mayo, J. Kitajewski, and C. Y. Wang**. 2001. Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription. J.Cell Biol **152**:87-96.

9. **Delhase, M., M. Hayakawa, Y. Chen, and M. Karin**. 1999. Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation [see comments]. Science **284**:309-313.

10. **Deng, J., S. A. Miller, H. Y. Wang, W. Xia, Y. Wen, B. P. Zhou, Y. Li, S. Y. Lin, and M. C. Hung**. 2002. beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. Cancer Cell **2**:323-334.

11. **Devin, A., Y. Lin, S. Yamaoka, Z. Li, M. Karin, and Z. Liu**. 2001. The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. Mol.Cell Biol **21**:3986-3994.

12. **Ghosh, S. and M. Karin**. 2002. Missing pieces in the NF-kappaB puzzle. Cell **109 Suppl**:S81-S96.

13. **Ghosh, S., M. J. May, and E. B. Kopp**. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu.Rev Immunol. **16**:225-260.

14. **Guger, K. A. and B. M. Gumbiner**. 2000. A mode of regulation of beta-catenin signaling activity in Xenopus embryos independent of its levels. Dev.Biol **223**:441-448.

15. **Guger, K. A. and B. M. Gumbiner**. 2000. A mode of regulation of beta-catenin signaling activity in Xenopus embryos independent of its levels. Dev.Biol **223**:441-448.

16. **Henderson, B. R.** 2000. Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat.Cell Biol **2**:653-660.

17. **Hewitt, R. E., A. McMarlin, D. Kleiner, R. Wersto, P. Martin, M. Tsokos, G. W. Stamp, W. G. Stetler-Stevenson, and M. Tsoskas**. 2000. Validation of a model of colon cancer progression. J Pathol. **192**:446-454.

18. **Hinoi, T., H. Yamamoto, M. Kishida, S. Takada, S. Kishida, and A. Kikuchi**. 2000. Complex formation of adenomatous polyposis coli gene product and axin facilitates glycogen synthase kinase-3 beta-dependent phosphorylation of beta-catenin and down-regulates beta-catenin. J Biol Chem **275**:34399-34406.

19. **Hoeflich, K. P., J. Luo, E. A. Rubie, M. S. Tsao, O. Jin, and J. R. Woodgett**. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation [In Process Citation]. Nature 2000.Jul.6.;406.(6791.):86.-90. **406**:86-90.

20. **Hu, Y., V. Baud, M. Delhase, P. Zhang, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin**. 1999. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase [see comments]. Science **284**:316-320.

21. **Hu, Y., V. Baud, T. Oga, K. I. Kim, K. Yoshida, and M. Karin**. 2001. IKKalpha controls formation of the epidermis independently of NF-kappaB. Nature **410**:710-714.

22. **Johns, L. D., T. Sarr, and G. E. Ranges**. 1994. Inhibition of ceramide pathway does not affect ability of TNF-alpha to activate nuclear factor-kappa B. J Immunol. **152**:5877-5882.

23. **Karin, M.** 1999. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene **18**:6867-6874.

24. **Kawahara, K., T. Morishita, T. Nakamura, F. Hamada, K. Toyoshima, and T. Akiyama**. 2000. Down-regulation of beta-catenin by the colorectal tumor suppressor APC requires association with Axin and beta-catenin. J Biol Chem **275**:8369-8374.

25. **Kim, J. S., H. Crooks, A. Foxworth, and T. Waldman**. 2002. Proof-of-principle: oncogenic beta-catenin is a valid molecular target for the development of pharmacological inhibitors. Mol.Cancer Ther. **1**:1355-1359.

26. **Kitagawa, M., S. Hatakeyama, M. Shirane, M. Matsumoto, N. Ishida, K. Hattori, I. Nakamichi, A. Kikuchi, and K. Nakayama**. 1999. An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. EMBO J. **18**:2401-2410.

27. **Lamberti, C., K. M. Lin, Y. Yamamoto, U. Verma, I. M. Verma, S. Byers, and R. B. Gaynor**. 2001. Regulation of beta-catenin function by the IkappaB kinases. J Biol Chem **276**:42276-42286.

28. **Landesman-Bollag, E., R. Romieu-Mourez, D. H. Song, G. E. Sonenshein, R. D. Cardiff, and D. C. Seldin**. 2001. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene **20**:3247-3257.

29. **Landesman-Bollag, E., D. H. Song, R. Romieu-Mourez, D. J. Sussman, R. D. Cardiff, G. E. Sonenshein, and D. C. Seldin**. 2001. Protein kinase CK2: signaling and tumorigenesis in the mammary gland. Mol.Cell Biochem. **227**:153-165.

30. **Li, Q., Q. Lu, J. Y. Hwang, D. Buscher, K. F. Lee, J. C. Izpisua-Belmonte, and I. M. Verma**. 1999. IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev. **13**:1322-1328.

31. **Li, Y., J. Kang, J. Friedman, L. Tarassishin, J. Ye, A. Kovalenko, D. Wallach, and M. S. Horwitz**. 1999. Identification of a cell protein (FIP-3) as a modulator of NF-kappaB activity and as a target of an adenovirus inhibitor of tumor necrosis factor alpha-induced apoptosis. Proc.Natl Acad.Sci U.S.A **96**:1042-1047.

32. **Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin**. 1999. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J.Exp.Med. **189**:1839-1845.

33. **Lin, S. Y., W. Xia, J. C. Wang, K. Y. Kwong, B. Spohn, Y. Wen, R. G. Pestell, and M. C. Hung**. 2000. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc.Natl.Acad.Sci.U.S.A **97**:4262-4266.

34. **Masui, O., Y. Ueda, A. Tsumura, M. Koyanagi, M. Hijikata, and K. Shimotohno**. 2002. RelA suppresses the Wnt/beta-catenin pathway without exerting trans-acting transcriptional ability. Int J Mol.Med **9**:489-493.

35. **Mercurio, F., B. W. Murray, A. Shevchenko, B. L. Bennett, D. B. Young, J. W. Li, G. Pascual, A. Motiwala, H. Zhu, M. Mann, and A. M. Manning**. 1999. IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol.Cell Biol **19**:1526-1538.

36. **Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. Li, D. B. Young, M. Barbosa, M. Mann, A. Manning, and A. Rao**. 1997. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF- kappaB activation [see comments]. Science **278**:860-866.

37. **Nakamura, T., F. Hamada, T. Ishidate, K. Anai, K. Kawahara, K. Toyoshima, and T. Akiyama**. 1998. Axin, an inhibitor of the Wnt signalling pathway, interacts with beta- catenin, GSK-3beta and APC and reduces the beta-catenin level. Genes Cells **3**:395-403.

38. **Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner**. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature **401**:82-85.

39. **Prajapati, S. and R. B. Gaynor**. 2002. Regulation of Ikappa B kinase (IKK)gamma /NEMO function by IKKbeta -mediated phosphorylation. J Biol Chem **277**:24331-24339.

40. **Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin**. 1998. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex [see comments]. Nature **395**:297-300.

41. **Russo, M. P., B. L. Bennett, A. M. Manning, D. A. Brenner, and C. Jobin**. 2002. Differential requirement for NF-kappaB-inducing kinase in the induction of NF-kappaB by IL-1beta, TNF-alpha, and Fas. Am J Physiol Cell Physiol **283**:C347-C357.

42. **Sadot, E., M. Conacci-Sorrell, J. Zhurinsky, D. Shnizer, Z. Lando, D. Zharhary, Z. Kam, A. Ben Ze'ev, and B. Geiger**. 2002. Regulation of S33/S37 phosphorylated beta-catenin in normal and transformed cells. J Cell Sci **115**:2771-2780.

43. **Sadot, E., M. Conacci-Sorrell, J. Zhurinsky, D. Shnizer, Z. Lando, D. Zharhary, Z. Kam, A. Ben Ze'ev, and B. Geiger**. 2002. Regulation of S33/S37 phosphorylated beta-catenin in normal and transformed cells. J Cell Sci **115**:2771-2780.

44. **Sakanaka, C.** 2002. Phosphorylation and regulation of beta-catenin by casein kinase I epsilon. J Biochem.(Tokyo) **132**:697-703.

45. **Schwabe, R. F. and D. A. Brenner**. 2002. Role of glycogen synthase kinase-3 in TNF-alpha-induced NF-kappaB activation and apoptosis in hepatocytes. Am J Physiol Gastrointest.Liver Physiol **283**:G204-G211.

46. **Shtutman, M., J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell, and Z. Ben**. 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc.Natl.Acad.Sci.U.S.A. **96**:5522-5527.

47. **Staal, F. J., M. M. Noort, G. J. Strous, and H. C. Clevers**. 2002. Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. EMBO Rep. **3**:63-68.

48. **Takeda, K., O. Takeuchi, T. Tsujimura, S. Itami, O. Adachi, T. Kawai, H. Sanjo, K. Yoshikawa, N. Terada, and S. Akira**. 1999. Limb and skin abnormalities in mice lacking IKKalpha [see comments]. Science **284**:313-316.

49. **van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Louriero, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin, and H. Clevers**. 1997. Armadillo co-activates transcription driven by the product of the drosophila segment polarity gene dTCF. Cell **88**:789-799.

50. **van Noort, M., J. Meeldijk, Z. R. van der, O. Destree, and H. Clevers**. 2002. Wnt signaling controls the phosphorylation status of beta-catenin. J Biol Chem **277**:17901-17905.

51. **van Noort, M., W. M. van de, and H. Clevers**. 2002. Identification of two novel regulated serines in the N terminus of beta-catenin. Exp.Cell Res **276**:264-272.

52. **Vermeulen, L., G. De Wilde, S. Notebaert, W. Vanden Berghe, and G. Haegeman**. 2002. Regulation of the transcriptional activity of the nuclear factor-kappaB p65 subunit. Biochem.Pharmacol. **64**:963-970.

53. **Webster, G. A. and N. D. Perkins**. 1999. Transcriptional cross talk between NF-kappaB and p53. Mol.Cell Biol **19**:3485-3495.

54. **Woronicz, J. D., X. Gao, Z. Cao, M. Rothe, and D. V. Goeddel**. 1997. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. Science **278**:866-869.

55. **Yamamoto, Y., D. W. Kim, Y. T. Kwak, S. Prajapati, U. Verma, and R. B. Gaynor**. 2001. IKKgamma /NEMO facilitates the recruitment of the IkappaB proteins into the IkappaB kinase complex. J Biol Chem **276**:36327-36336.

56. **Yamaoka, S., G. Courtois, C. Bessia, S. T. Whiteside, R. Weil, F. Agou, H. E. Kirk, R. J. Kay, and A. Israel**. 1998. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell **93**:1231-1240.

57. **Zandi, E. and M. Karin**. 1999. Bridging the gap: composition, regulation, and physiological function of the IkappaB kinase complex. Mol.Cell Biol **19**:4547-4551.

58. **Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin**. 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell **91**:243-252.

59. **Zhu, A. J. and F. M. Watt**. 1999. beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. Development **126**:2285-2298.

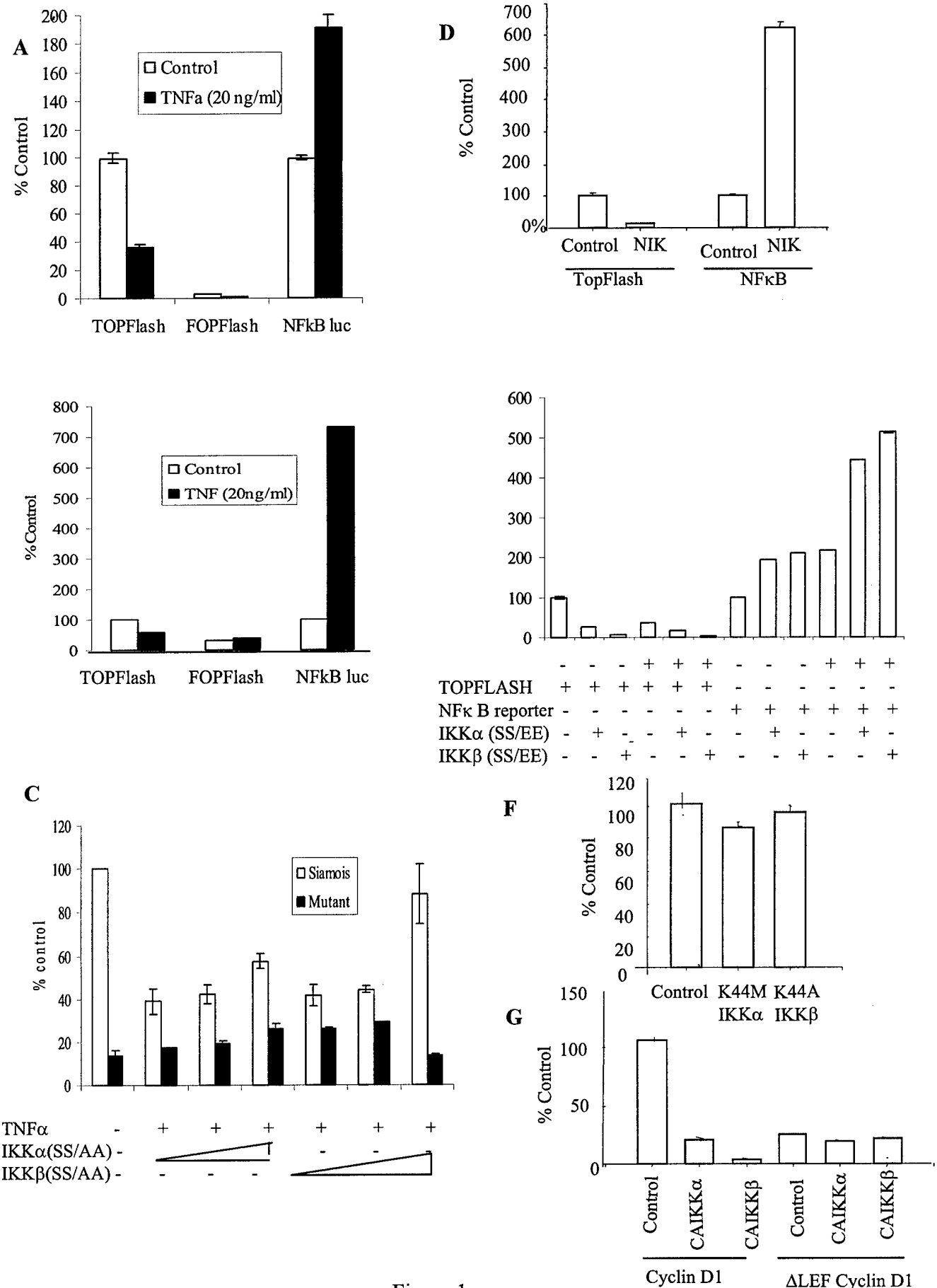

A
% Control
Control
TNFa (20 ng/ml)
TOPFlash
FOPFlash
NFkB luc
D
% Control
Control
NIK
TopFlash
NFκB
% Control
Control
TNF (20ng/ml)
TOPFlash
FOPFlash
NFkB luc
TOPFLASH
NFκ B reporter
IKKα (SS/EE)
IKKβ (SS/EE)
C
% control
Siamois
Mutant
TNFα
IKKα(SS/AA)
IKKβ(SS/AA)
F
% Control
Control
K44M IKKα
K44A IKKβ
G
% Control
Control
CAIKKα
CAIKKβ
Cyclin D1
ΔLEF Cyclin D1

Figure 1

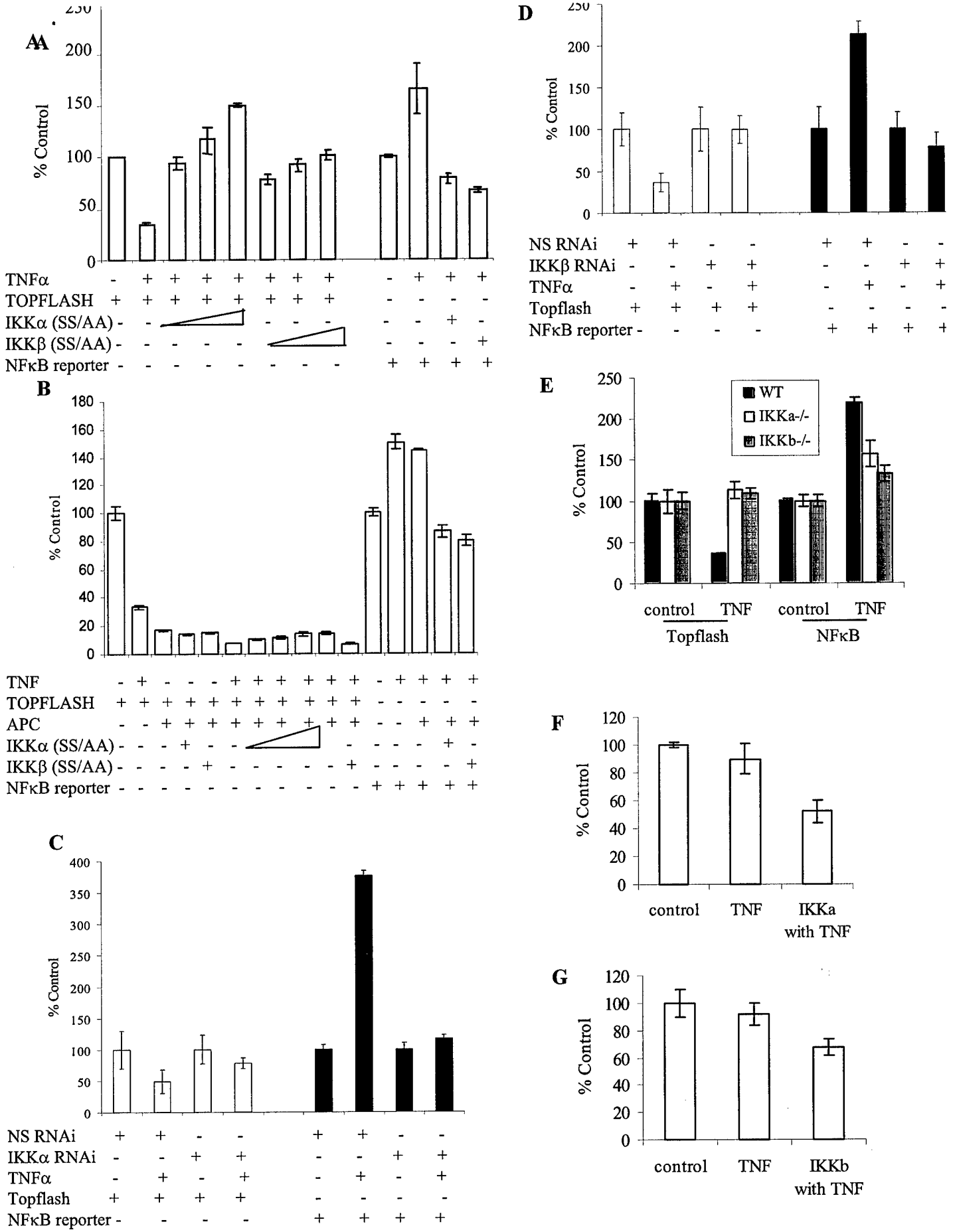

AA
% Control
TNFα
TOPFLASH
IKKα (SS/AA)
IKKβ (SS/AA)
NFκB reporter
B
% Control
TNF
TOPFLASH
APC
IKKα (SS/AA)
IKKβ (SS/AA)
NFκB reporter
C
% Control
NS RNAi
IKKα RNAi
TNFα
Topflash
NFκB reporter
D
% Control
NS RNAi
IKKβ RNAi
TNFα
Topflash
NFκB reporter
E
WT
IKKa-/-
IKKb-/-
% Control
control
TNF
Topflash
NFκB
F
% Control
control
TNF
IKKa with TNF
G
% Control
control
TNF
IKKb with TNF

Figure 2

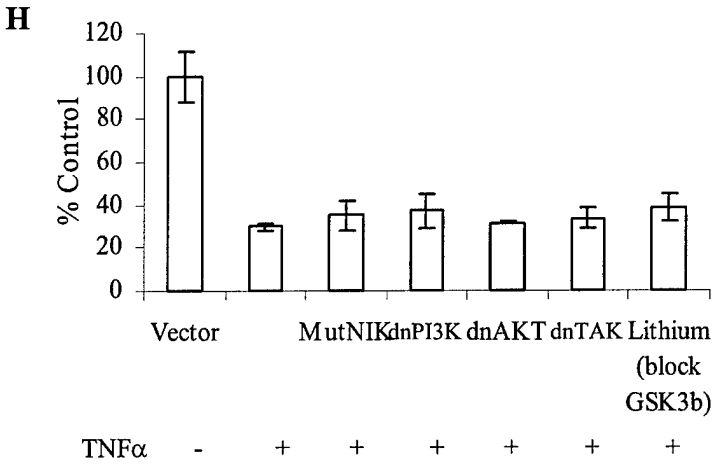
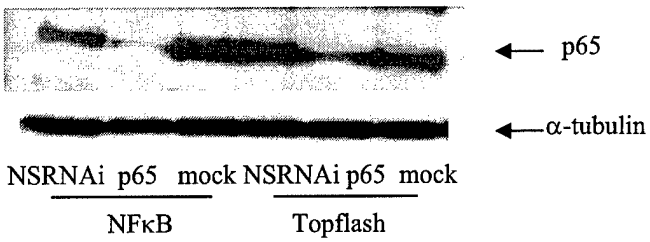
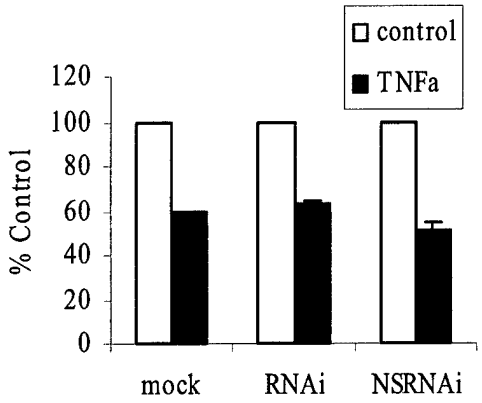

H
120
100
80
60
40
20
0
% Control
Vector
MutNIK
dnPI3K
dnAKT
dnTAK
Lithium (block GSK3b)
TNFα - + + + + + +

Figure 2

A


p65
α-tubulin
NSRNAi p65 mock NSRNAi p65 mock
NFκB
Topflash

B


control
TNFa
% Control
120
100
80
60
40
20
0
mock
RNAi
NSRNAi

C

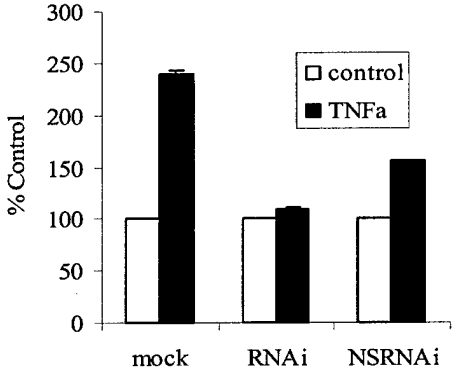

control
TNFa
% Control
300
250
200
150
100
50
0
mock
RNAi
NSRNAi

Figure 3

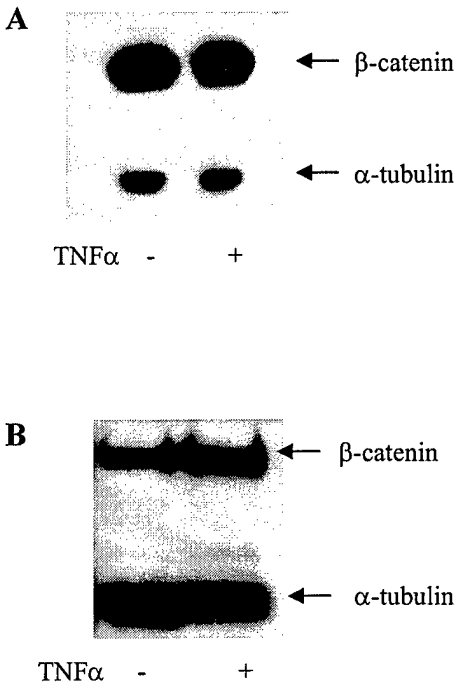


Figure 4

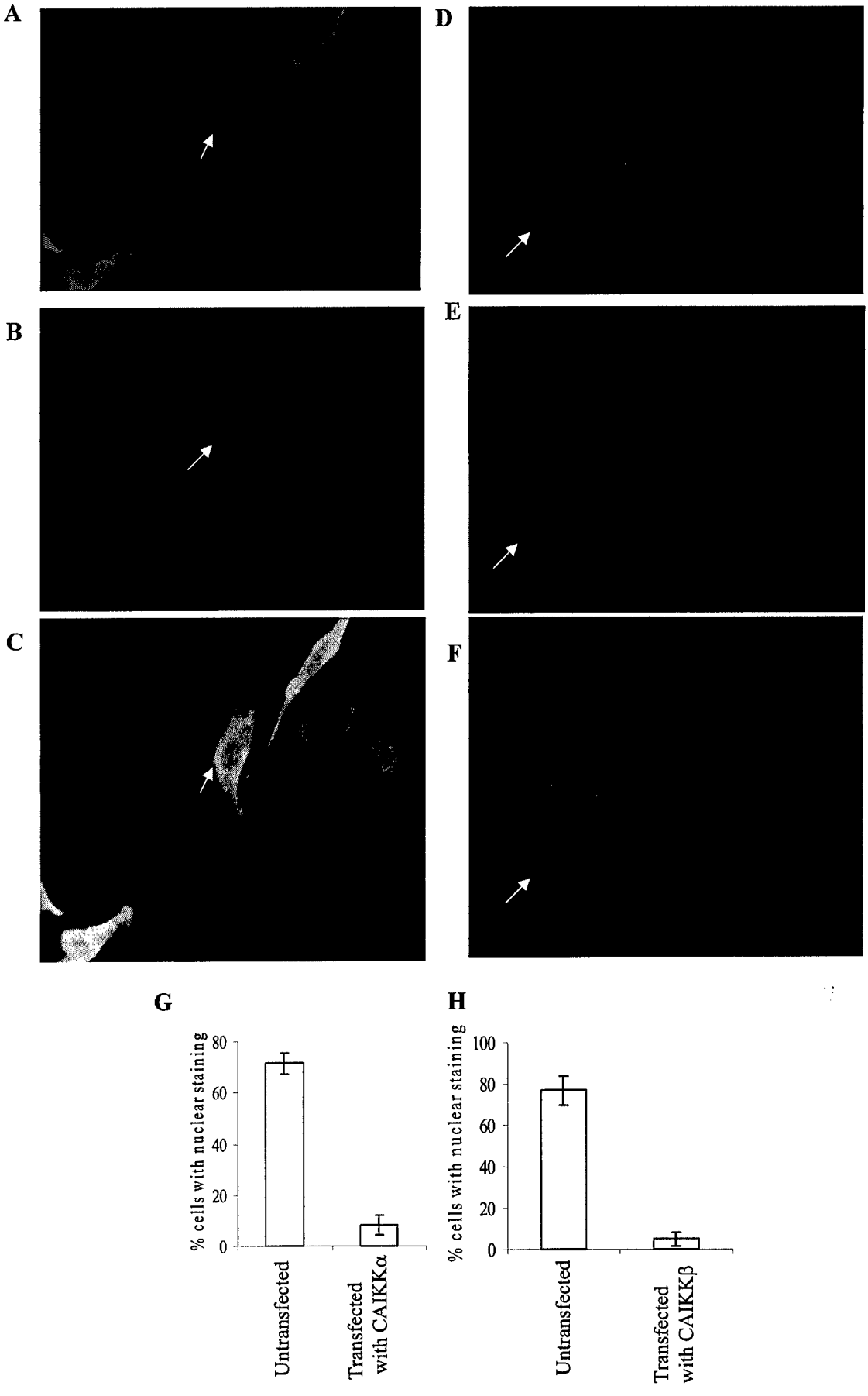
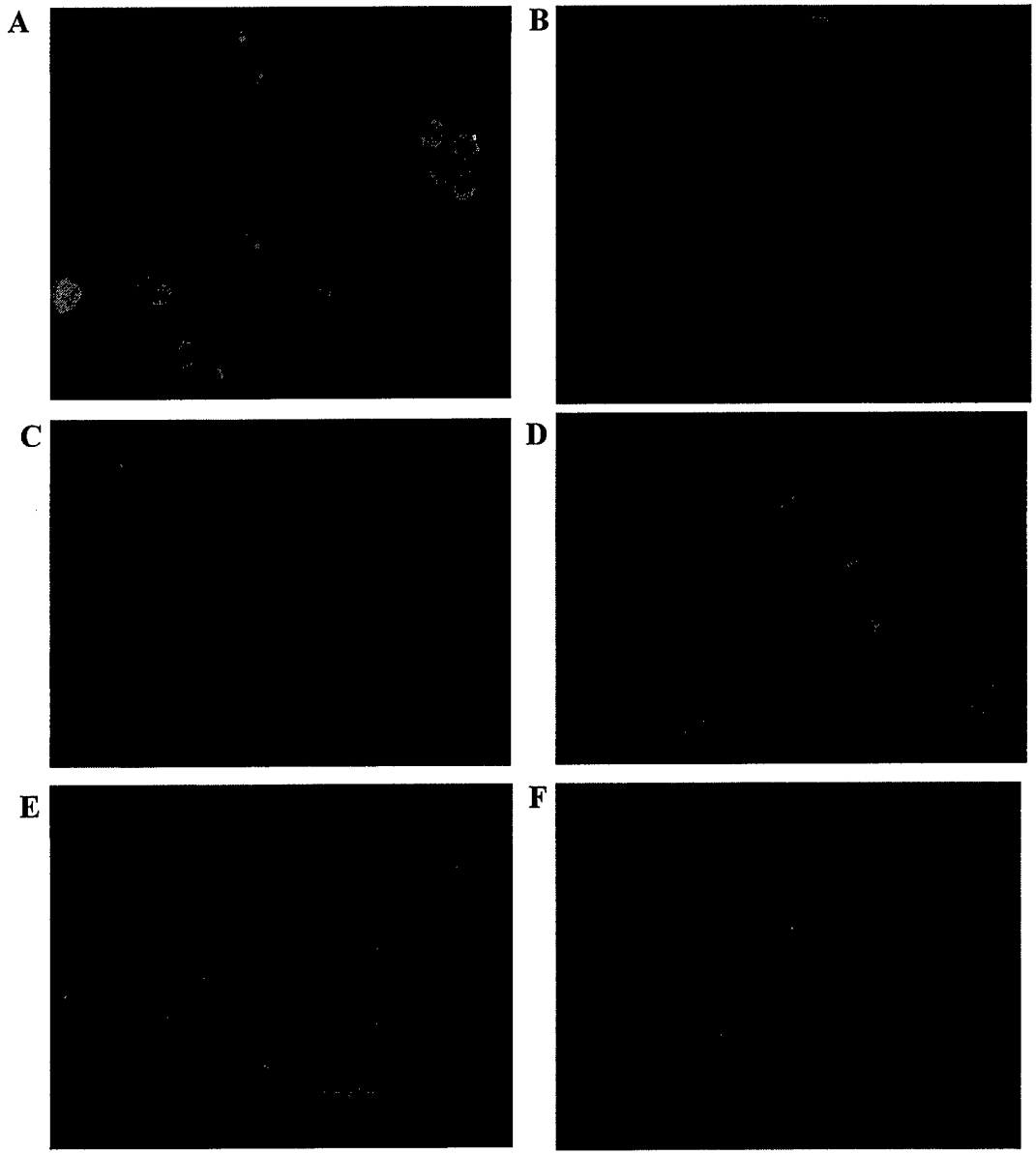

A
B
C
D
E
F
G
% cells with nuclear staining
0
20
40
60
80
Untransfected
Transfected with CAIKKα
H
% cells with nuclear staining
0
20
40
60
80
100
Untransfected
Transfected with CAIKKβ

Figure 5


A
B
C
D
E
F

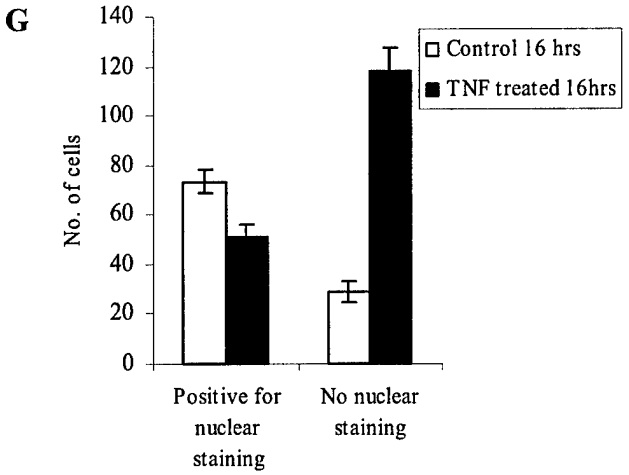
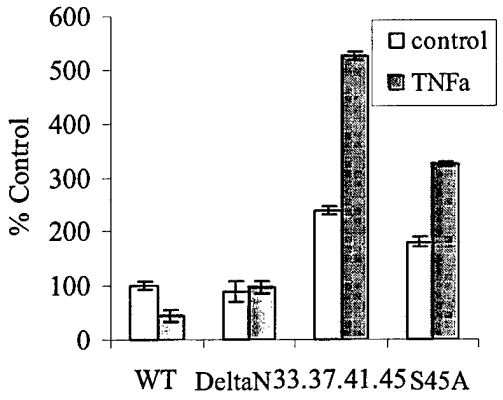
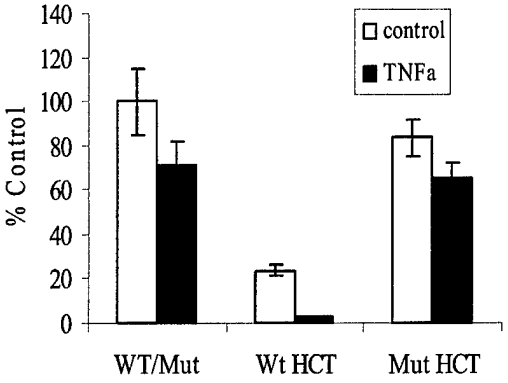

G
140
120
100
80
60
40
20
0
No. of cells
Control 16 hrs
TNF treated 16hrs
Positive for nuclear staining
No nuclear staining

Figure 6

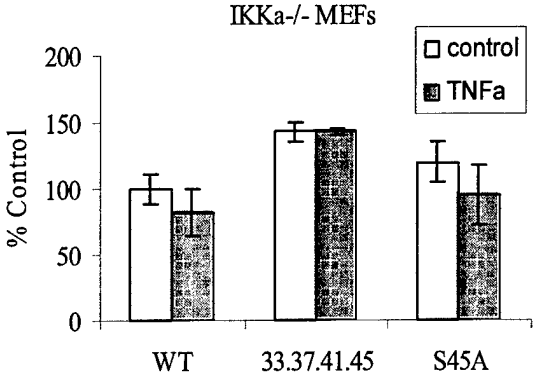
A



D



B



C

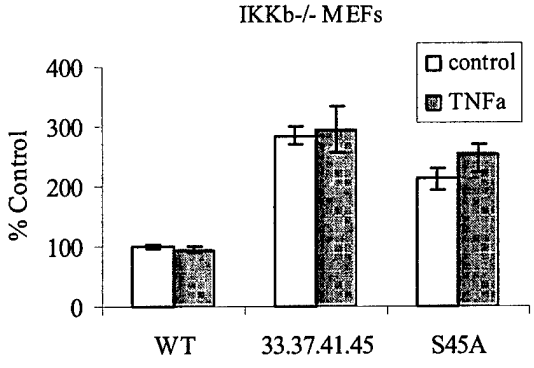


Figure 7

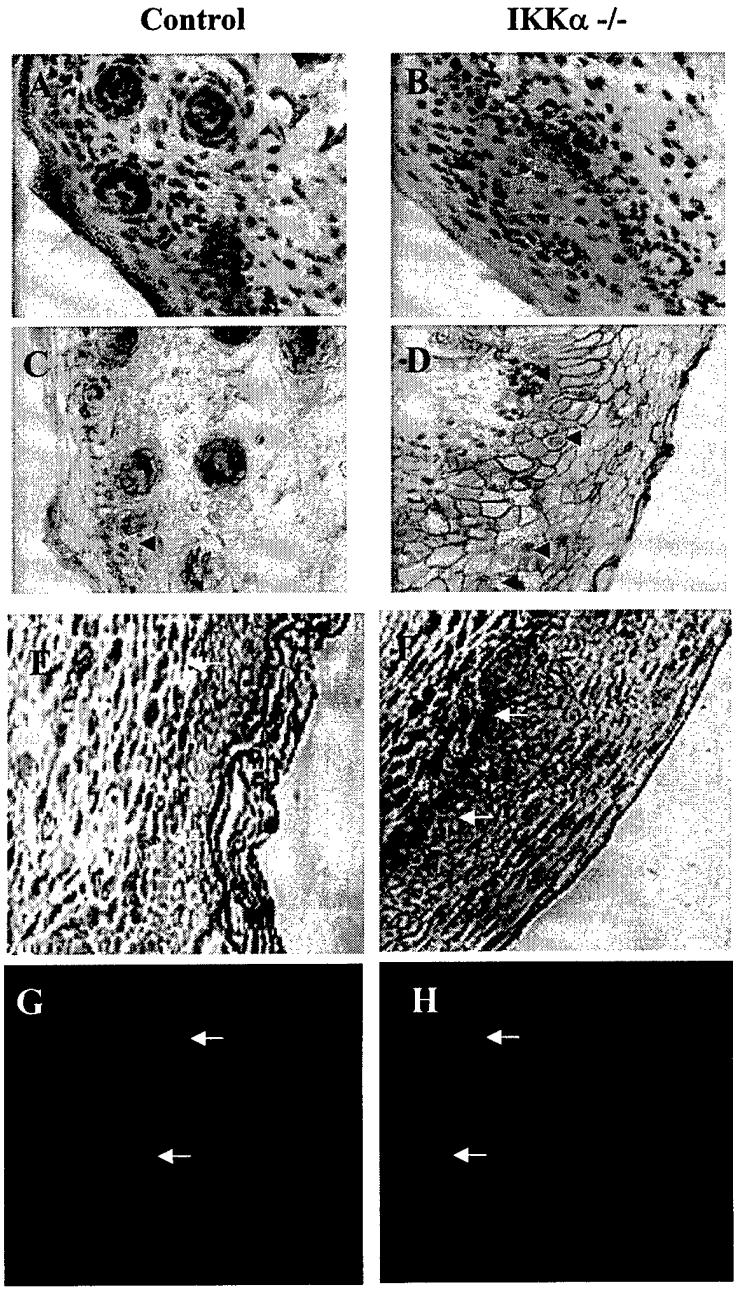


Figure 8

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 23, Issue of June 4, pp. 16641–16645, 1999
Printed in U.S.A.

The Ubiquitin-Proteasome Pathway and Serine Kinase Activity Modulate Adenomatous Polyposis Coli Protein-mediated Regulation of β-Catenin-Lymphocyte Enhancer-binding Factor Signaling*

(Received for publication, January 21, 1999)

Vijayasurian Easwaran‡, Virginia Song‡, Paul Polakis§, and Stephen Byers‡¶

From the ‡Department of Cell Biology, Georgetown University School of Medicine and Lombardi Cancer Research Center, Washington, D. C. 20007 and §Onyx Pharmaceuticals, Richmond, California 94806

The tumor suppressor function of the adenomatous polyposis coli protein (APC) depends, in part, on its ability to bind and regulate the multifunctional protein, β-catenin. β-Catenin binds the high mobility group box transcription factors, lymphocyte enhancer-binding factor (LEF) and T-cell factor, to directly regulate gene transcription. Using LEF reporter assays we find that APC-mediated down-regulation of β-catenin-LEF signaling is reversed by proteasomal inhibitors in a dose-dependent manner. APC down-regulates signaling induced by wild type β-catenin but not by the non-ubiquitinatable S37A mutant, β-catenin. Bisindoylmaleimide-type protein kinase C inhibitors, which prevent β-catenin ubiquitination, decrease the ability of APC to down-regulate β-catenin-LEF signaling. All these effects on LEF signaling are paralleled by changes in β-catenin protein levels. Lithium, an inhibitor of glycogen synthase kinase-3β, does not alter the ability of APC to down-regulate β-catenin protein and β-catenin-LEF signaling in the colon cancer cells that were tested. These results point to a role for β-catenin ubiquitination, proteasomal degradation, and potentially a serine kinase other than glycogen synthase kinase-3β in the tumor-suppressive actions of APC.

Mutations in the tumor suppressor adenomatous polyposis coli (APC)[1] gene are responsible for tumors that arise in both familial adenomatous polyposis and sporadic colon cancers (1–7). APC mutations are almost always truncating, giving rise to proteins lacking C termini (6, 8, 9). Efforts to understand how these mutations contribute to cancer have focused on the ability of APC to bind and subsequently down-regulate the cytoplasmic levels of β-catenin (10–13).

β-Catenin is a multifunctional protein that participates in cadherin-mediated cell-cell adhesion and in transduction of the Wnt growth factor signal that regulates development (14, 15). Activation of the Wnt growth factor signaling cascade results in the inhibition of the serine/threonine kinase, GSK-3β, and in response, β-catenin accumulates in the cytoplasm (16–18). At elevated cytoplasmic levels, β-catenin translocates to the nucleus, interacts with the high mobility group box transcriptional activator lymphocyte enhancer-binding factor (LEF)/T-cell factor, and directly regulates gene expression (19–22). Mutations that stabilize β-catenin protein are likely to be oncogenic, although this has not been proven directly (23).

The mechanism of APC-mediated β-catenin regulation is unknown. Recently, β-catenin was shown to be regulated at the level of protein stability via proteasomal degradation (24, 25). Proteins targeted for degradation by the ubiquitin-proteasome system are first tagged with multiple copies of the small protein ubiquitin by highly regulated ubiquitination machinery (27). Polyubiquitinated proteins are recognized and rapidly degraded by the proteasome, a large multisubunit proteolytic complex. Proteasomal degradation plays a critical role in the rapid elimination of many important regulatory proteins, *e.g.* cyclins and transcriptional activators like NFκB-IκB (28). Proteins regulated via proteasomal degradation can be specifically studied using the well characterized proteasome-specific peptidyl-aldehyde inhibitors (29, 30).

APC-mediated tumorigenesis might depend, in part, on its ability to regulate β-catenin signaling (26). In this report, we show that the ubiquitin-proteasome pathway and the activity of a serine kinase other than GSK-3β modulate APC-mediated regulation of β-catenin-LEF signaling.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cells—ALLN, ALLM, lactacystin-β lactone, and MG-132 were purchased from Calbiochem. GF-109203X was purchased from Roche Molecular Biochemicals. Ro31-8220 was a gift from Dr. Robert Glazer. The monoclonal anti-β-catenin antibody (Clone 14) and the anti-FLAG™ antibody were purchased from Transduction Laboratories, Lexington, KY and Eastman Kodak Co., respectively. Affinity-purified rabbit polyclonal anti-APC2 and anti-APC3 antibodies (12) were generously provided by Dr. Paul Polakis (Onyx Pharmaceuticals). Affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies were purchased from Kirkegaard and Perry Laboratories. The SW480 and CACO-2 colon cancer cell lines were acquired from the ATCC and maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 1% penicillin/streptomycin.

Transfections and LEF-Luciferase Reporter Assays—Cells were seeded in 12-well plates at 1×10^5 cells/well. The following day cells were transiently transfected with 1 μg of APC constructs and 0.4 μg of the LEF reporter, pTOPFLASH (optimal motif), or pFOPFLASH (mutant motif) (31), and 0.008 μg of pCMV-*Renilla* luciferase (Promega) per well, using LipofectAMINE-Plus reagent according to the manufacturer's instructions (Life Technologies, Inc.) for 5 h. In experiments designed to monitor the effect of APC on β-catenin protein, 0.3 μg of FLAG-tagged WT or S37A β-catenin (25) was cotransfected with 0.6 μg of empty vector or APC constructs. This approach facilitated analysis of only the transfected cells, using anti-FLAG antibodies.

Cells were treated with indicated levels of the inhibitors for 12–24 h. Luciferase activity was monitored using the dual luciferase assay sys-

* This work was supported by Grants DAMD1794J-4171 (to V. E.) and DAMD17-98-1-8089 (to S. B.) from the Department of Defense. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: E415 The Research Building, GUMC, 3970 Reservoir Rd., NW, Washington, D. C. 20007. Tel.: 202-687-1813; Fax: 202-687-7505; E-mail: byerss@gunet.georgetown.edu.

[1] The abbreviations used are: APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3β; LEF, lymphocyte enhancer-binding factor; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ALLM, *N*-acetyl-Leu-Leu-methional; WT, wild type; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DAG, diacylglycerol; NKκB, nuclear factor κB; IκB, inhibitor of NFκB.

tem (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection efficiency and potential toxicity of treatments using the constitutively expressed pCMV-*Renilla* luciferase. The specificity of APC-mediated effects on LEF reporters was confirmed using pFOPFLASH, which harbors mutated LEF binding sites (31), and an unrelated AP-1 reporter (32).

Immunological Procedures—Double immunofluorescent staining for APC and β-catenin was performed according to Munemitsu *et al.* (11, 40). In experiments where FLAG-tagged β-catenin was cotransfected with APC, anti-FLAG™ antibodies (Kodak) were used to detect the exogenous β-catenin.

RESULTS AND DISCUSSION

APC-mediated Down-regulation of β-Catenin-LEF Signaling Is Reversed by Proteasomal Inhibitors—In the SW480 colon cancer cell line, which produces only a mutant APC protein containing amino acids 1–1337 of the complete 2843-amino acid sequence, overexpression of WT APC or deletion construct APC 25 (amino acids 1342–2075), but not APC 3 (amino acids 2130–2843) (Fig. 1*A*), can effect a posttranslational down-regulation of β-catenin (11, 26). We tested the hypothesis that APC effects the down-regulation of β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation. SW480 cells were transiently transfected with various APC deletion constructs (Fig. 1*A*) and treated with proteasomal inhibitors, and β-catenin-LEF signaling was assayed using LEF reporters (31). Fig. 1*B* shows that the APC-mediated down-regulation of β-catenin-LEF signaling is reversed by a panel of proteasomal inhibitors including ALLN, lactacystin-β lactone, and MG-132, but not Me_2SO (vehicle) or ALLM (calpain inhibitor II), that effectively inhibits calpain proteases but has a 100-fold lower potency as a proteasomal inhibitor. The specificity of APC-mediated effects on LEF reporters was confirmed using pFOPFLASH, which harbors mutated LEF binding sites, and an unrelated AP-1 reporter, neither of which was influenced by APC (31, 32). The proteasomal inhibitor ALLN reverses the APC- mediated down-regulation of β-catenin-LEF signaling in a dose-dependent manner (Fig. 1*C*). The effects of APC 25 can be completely reversed by the proteasomal inhibitor ALLN, and the effects of WT APC can be restored to 50–60% of control values. However, the full-length WT APC construct, and not the APC 25 deletion construct, was used for all immunostaining experiments because it was more physiologically relevant (incorporating all the functional domains). SW480 cells were transfected with empty vector or WT APC and were treated with Me_2SO (vehicle) or the proteasomal inhibitors ALLN or lactacystin-β lactone. Double immunofluorescent staining for APC (Fig. 2, *A*, *C*, and *E*) and β-catenin (Fig. 2, *B*, *D*, and *F*) shows that the APC induced reduction in β-catenin protein (Fig. 2, *A* and *B*) is reversed by proteasomal inhibitors ALLN (Fig. 2, *C* and *D*) and lactacystin-β lactone (Fig. 2,*E* and *F*).

APC Down-regulates WT β-Catenin but Not the Non-ubiquitinatable S37A Mutant Form of β-Catenin-induced LEF Signaling—Mutation of a single serine residue (S37A) within the ubiquitination-targeting sequence prevents β-catenin ubiquitination (25). Serine mutations in the ubiquitin-targeting sequence of β-catenin occur in a number of different cancers (33–38). At least one of these, S37A, is a stabilizing mutation that renders β-catenin resistant to ubiquitination (25). If indeed APC regulates β-catenin-LEF signaling by targeting β-catenin for proteasomal degradation, then it should not be able to down-regulate the non-ubiquitinatable S37A mutant β-catenin protein or the LEF signaling induced by this stable form of β-catenin. To test this hypothesis, vector, FLAG-tagged WT, or S37A mutant β-catenin constructs were cotransfected with vector or WT APC and the LEF reporters into SW480 cells. β-Catenin-LEF signaling was monitored by assaying LEF

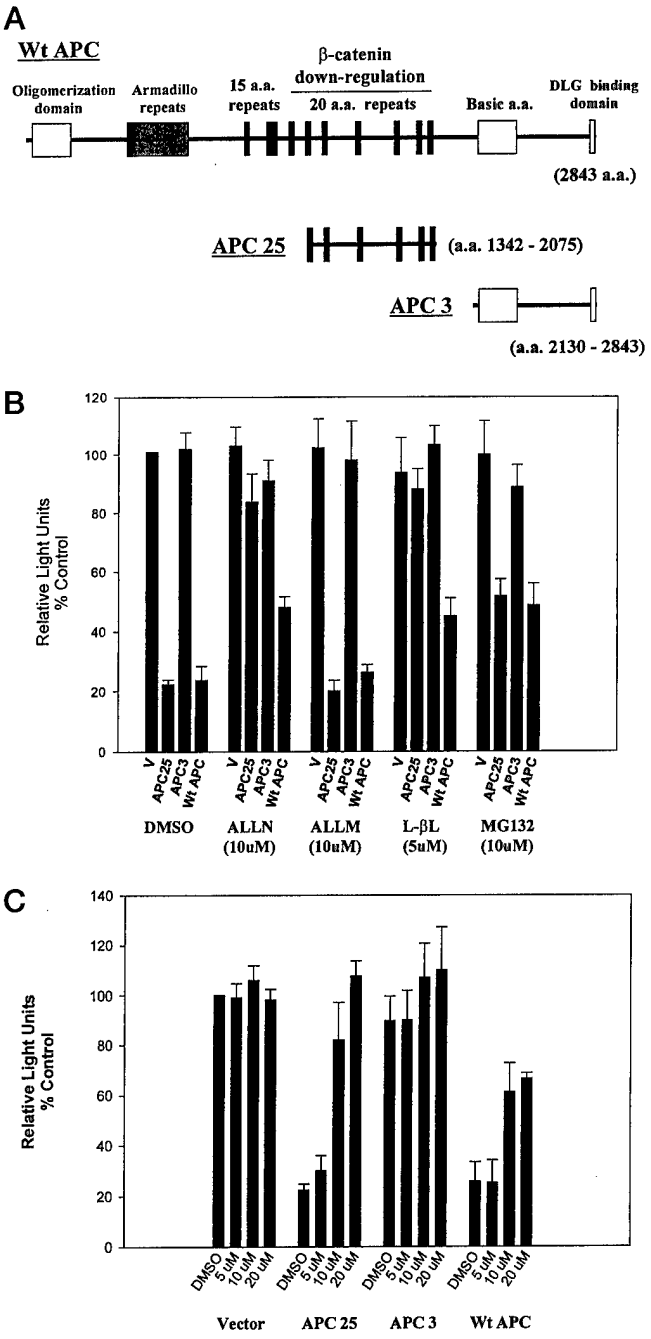


FIG. 1. *A*, the structure of WT APC and APC deletion constructs (26); *B*, APC-mediated down-regulation of β-catenin- LEF signaling is reversed by proteasomal inhibitors. SW480 cells were transiently transfected with various APC constructs, using LipofectAMINE-Plus reagent (Life Technologies, Inc.). 12 h posttransfection, the cells were treated with proteasomal inhibitors ALLN, lactacystin-β lactone, and MG-132 or with Me_2SO (*DMSO*, vehicle) and ALLM (calpain inhibitor II) for 12 h. β-Catenin-LEF signaling was assayed using the LEF reporters pTOPFLASH (and pFOPFLASH; data not shown) (31). Raw data were normalized for transfection efficiency and potential toxicity of treatments, using pCMV-*Renilla* luciferase and the dual luciferase assay system (Promega). The experiment was repeated at least three times, with each treatment repeated in triplicate. *Error bars* represent S.D. *C*, APC-mediated down-regulation of β-catenin-LEF signaling is reversed by the proteasomal inhibitor, ALLN, in a dose-dependent manner. The transfections were performed as described in *B* and were followed by treatment with the various doses (μM) of the proteasomal inhibitor, ALLN. *a.a.*, amino acid(s); *DLG*, Discs Large protein.

reporter activity. Overexpression of both WT and S37A mutant forms of β-catenin increased the basal LEF reporter activity by about 30%, even against the background of high levels of en-

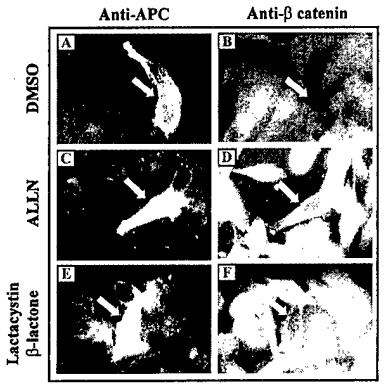


FIG. 2. **APC-mediated down-regulation of β-catenin protein is reversed by proteasomal inhibitors.** SW480 cells were transfected with WT APC and treated with Me_2SO (*DMSO*, *A* and *B*), 10 μM ALLN (*C* and *D*), or 5 μM lactacystin-β lactone (*E* and *F*). Double immunofluorescent staining for APC (*A*, *C*, and *E*) and β-catenin (*B*, *D*, and *F*) was performed according to Munemitsu *et al.* (11, 40).

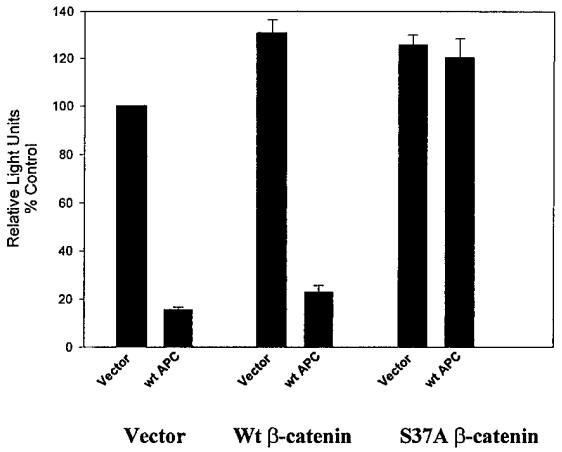


FIG. 3. **APC down-regulates LEF signaling induced by WT β-catenin but not by the non-ubiquitinatable S37A mutant β-catenin.** SW480 cells were transfected with empty vector or FLAG-tagged WT β-catenin or FLAG-tagged S37A β-catenin and empty vector or WT APC constructs, LEF reporters, and pCMV-*Renilla* luciferase. 24 h posttransfection, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

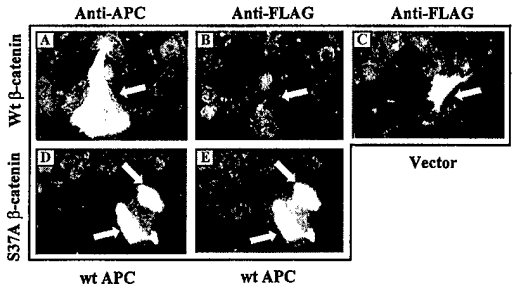


FIG. 4. **APC down-regulates WT β-catenin but not the non-ubiquitinatable S37A mutant β-catenin protein.** SW480 cells were transfected with FLAG-tagged WT β-catenin (*A*, *B*, and *C*) or FLAG-tagged S37A β-catenin (*D* and *E*) and WT APC constructs (*A*, *B*, *D*, and *E*) or empty vector (*C*). Double immunofluorescent staining for APC (*A* and *D*) and β-catenin (*B*, *C*, and *E*) were performed according to Munemitsu *et al.* (11, 40), except that the tranfected FLAG-tagged β-catenin was detected using anti-FLAG antibodies (Kodak).

dogenous β-catenin and β-catenin-LEF signaling in the SW480 cells. S37A β-catenin is more stable than WT β-catenin (in cells that actively degrade β-catenin, *e.g.* SKBR3 cells), but both forms increased LEF signaling by comparable levels in SW480 cells (which lack the ability to degrade β-catenin). Fig. 3 shows that APC down-regulates LEF signaling induced by WT β-catenin but not by the S37A mutant β-catenin. The ability of APC to down-regulate the cotransfected FLAG-tagged WT β-catenin and the S37A β-catenin protein levels was examined by double immunofluorescent staining using anti-APC antibodies and anti-FLAG antibodies (Kodak) (40). By double immunofluorescent staining for both the FLAG epitope and APC, we were able to monitor effects of APC specifically on the coexpressed forms of β-catenin. Fig. 4*A* (anti-APC) and Fig. 4*B* (anti-FLAG) show that WT APC effectively down-regulates WT β-catenin. Fig. 4*C* (anti-FLAG) shows that in concurrent transfections with empty vector and FLAG-tagged WT β-catenin, the FLAG-tagged WT β-catenin is expressed and the anti-FLAG antibody efficiently detects it. Fig. 4, *D* and *E* shows that APC does not down-regulate the S37A mutant β-catenin protein. These findings complement the observations of Munemitsu *et al.* (41) and Li *et al.* (42) that APC associates with but does not down-regulate β-catenin with an N-terminal deletion.

The Bisindoylmaleimide-type PKC Inhibitor GF-109203X Decreases the Ability of APC to Down-regulate LEF Signaling in a Dose-dependent Manner—PKC activity is required for Wnt-1 growth factor signaling to inhibit GSK-3β activity (18). TPA-induced down-regulation of diacylglycerol (DAG)-dependent PKCs prevents Wnt from inhibiting GSK-3β (18). However, our earlier studies demonstrate that neither the PKC inhibitor calphostin C nor TPA-induced down-regulation of PKCs stabilizes β-catenin (25). In contrast, the bisindoylmaleimide-type PKC inhibitor GF-109203X causes a dramatic accumulation of β-catenin in the cytoplasm (25). The bisindoylmaleimides inhibit both DAG-dependent and -independent PKC isoforms by competing with ATP for binding to the kinase, whereas calphostin C and long term TPA treatment inhibit only DAG-dependent PKC activities. The inhibitor profile implicates DAG-independent, atypical PKC activity in regulating β-catenin stability. These kinase(s) may offer a level of regulation distinct from the DAG-dependent PKC isoforms that regulate Wnt-dependent and GSK-3β-mediated β-catenin signaling (25).

The bisindoylmaleimide-type PKC inhibitor GF-109203X prevents β-catenin ubiquitination but does not inhibit GSK-3β (25). We tested the hypothesis that GF-109203X will inhibit the ability of APC to regulate β-catenin-LEF signaling. Fig. 5 shows that the PKC inhibitor GF-109203X decreases the ability of APC to down-regulate LEF signaling in a dose-dependent manner in SW480 cells. The changes in β-catenin-LEF signaling are paralleled by changes in β-catenin protein (Fig. 6). Similar results were obtained with another bisindoylmaleimide-type PKC inhibitor Ro31-8220 (data not shown).

Lithium (Li^+) Does Not Inhibit the Ability of APC to Down-regulate β-Catenin-LEF Signaling—Physiologically effective concentrations of Li^+ specifically and reversibly inhibit GSK-3β activity *in vitro* and *in vivo* and can mimic the effects of Wnt signaling on β-catenin in mammalian cells (43–46). Treatment of breast cancer cell lines with lithium results in the accumulation of the cytoplasmic signaling pool of β-catenin (25). Axin, the recently described product of the mouse *Fused* locus, forms a complex with GSK-3β, β-catenin, and APC (47). Axin promotes GSK-3β-dependent phosphorylation of β-catenin and may therefore help target β-catenin for degradation (48). However, overexpression of Axin inhibits β-catenin-LEF signaling in SW480 colon cancer cells in the absence of functional, WT APC. It is not known if APC promotes GSK-3β-dependent phosphorylation of β-catenin. Rubinfeld *et al.* (49) have shown that the APC protein is phosphorylated by GSK-3β *in vitro* and suggest that this phosphorylation event is linked to β-catenin turnover. It has also been suggested that APC and Axin may regulate the degradation of β-catenin by different mechanisms (50).

We tested the hypothesis that Li^+ can inhibit the ability of

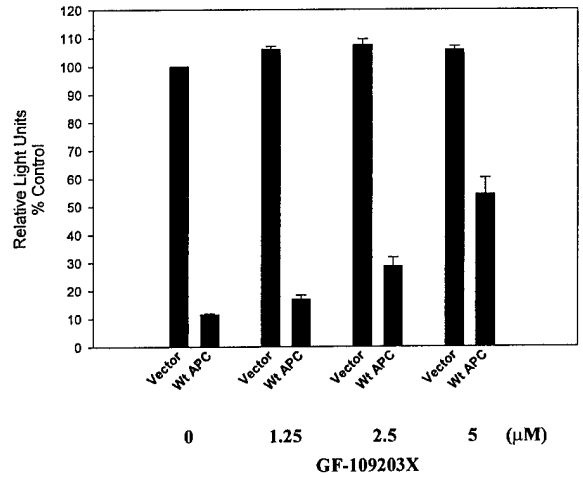


FIG. 5. **The bisindoylmaleimide-type PKC inhibitor, GF-109203X, which prevents β-catenin ubiquitination, inhibits APC-mediated down-regulation of β-catenin-LEF signaling in a dose-dependent manner.** SW480 cells were transfected with empty vector or WT APC, LEF reporters, and pCMV-*Renilla* luciferase. 12 h posttransfection, cells were treated with various concentrations of GF-109203X. 12 h later, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

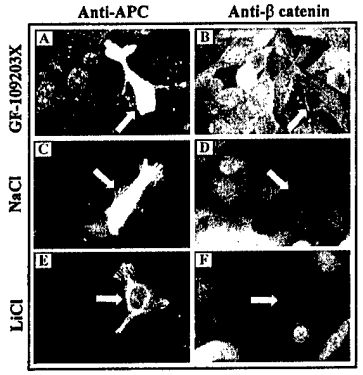


FIG. 6. **The bisindoylmaleimide-type PKC inhibitor, GF-109203X, but not lithium, reverses the APC-mediated down-regulation of β-catenin protein.** SW480 cells were transfected with WT APC and were treated with 5 μM GF-109203X (*A* and *B*) for 12 h as described in Fig. 5. 20 mM NaCl (*C* and *D*) or LiCl (*E* and IF) were added immediately following transfections and were present throughout the 24-h assay period to assure GSK-3β repression. Double immunofluorescent staining for APC (*A*, *C*, and *E*) and β-catenin (*B*, *D*, and *F*) was performed according to Munemitsu *et al.* (11, 40).

APC to down-regulate β-catenin-LEF signaling. The colon cancer cell line SW480 was transfected with empty vector or WT APC and treated with 10, 20, or 40 mM LiCl or NaCl for 24 h. The treatments were initiated immediately following the 5-h transfection period, and the cells were exposed to LiCl or NaCl throughout the 24-h assay period to assure GSK-3β repression. Fig. 6 shows that lithium does not alter the ability of WT APC to down-regulate β-catenin protein. Fig. 7 shows that lithium does not reverse the ability of WT APC to down-regulate LEF reporter activity in SW480 cells. Even at 40 mM lithium, a level well above that required to completely inhibit GSK-3β, exogenous WT APC continues to significantly down-regulate LEF reporter activity. These experiments were repeated in several different formats incorporating variations in the amount of WT APC transfected, duration of treatment with lithium, and timing of treatment initiation following transfections. Regardless of these variations, lithium does not inhibit the ability of exogenous APC to down-regulate β-catenin-LEF signaling in the colon cancer cells tested. Lithium treatment also leads to activation of AP-1-luciferase reporter activity in *Xenopus* embryos,

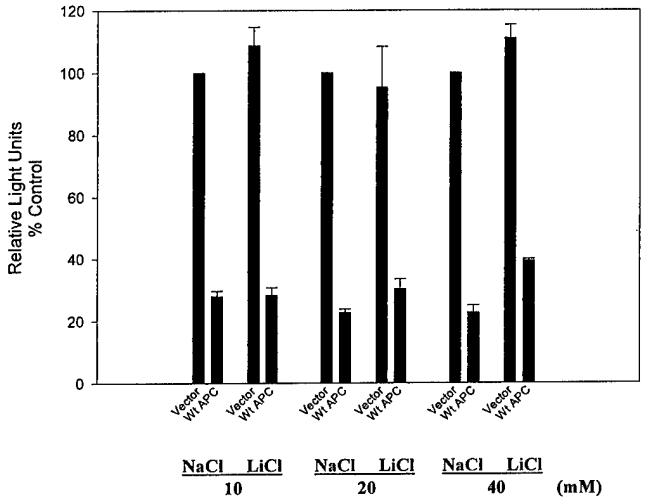


FIG. 7. **Lithium, an inhibitor of GSK-3β, does not significantly alter the ability of exogenous WT APC to down-regulate LEF reporter activity.** SW480 cells were transfected with empty vector or WT APC, LEF reporters, and pCMV-*Renilla* luciferase. Various concentrations of NaCl or LiCl were added immediately after transfection to assure GSK-3β repression. 24 h later, LEF reporter activity was monitored using the dual luciferase assay system (Promega).

consistent with previous observations that GSK-3β inhibits c-*jun* activity (46, 51). Concurrent AP-1 transactivation assays also confirmed that GSK-3β was inhibited in SW480 cells following treatment with lithium (data not shown). These results indicate that GSK-3β activity (the molecular target of lithium action, in the Wnt signaling cascade) is not required for the ability of exogenously expressed APC to down-regulate β-catenin. Recent data indicated that the role of GSK-3β may be to potentiate assembly of the APC·Axin·β-catenin complex (48). In our experiments, the high level of APC expressed in the transiently transfected cells may well drive complex assembly in the absence of GSK-3β activity. Indeed, in SKBR3 cells, lithium treatment causes the accumulation of cytoplasmic β-catenin and increases β-catenin-LEF signaling[2] (25).

Our observations suggest that one function of APC is to down-regulate β-catenin-LEF signaling via the ubiquitin-proteasome pathway. *In vitro* reconstitution experiments designed to explore β-catenin ubiquitination suggested the requirement of key components other than GSK-3β and APC.[2] During the course of this study there has been an explosion of data describing novel proteins, including Axin, Conductin, and Slimb·β-TrCP as regulators of β-catenin stability (47, 52–57). In *Drosophila,* loss of function of Slimb results in accumulation of high levels of Armadillo and the ectopic expression of Wg-responsive genes (56). Recently, the receptor component of the IκB·ubiquitin ligase complex has been identified as a member of the Slimb·β-TrCP family (39). Considering the increasing number of similarities between the regulation of IκB and β-catenin (25), it is tempting to speculate that like IκB, β-catenin ubiquitination occurs in a multiprotein complex that includes kinases, ubiquitin-conjugating enzymes, and co-factors. Context-dependent potentiation of this complex by GSK-3β and other serine kinase(s) may be regulated by DAG-dependent and -independent PKC activity, respectively. The challenge for future studies will be to determine the exact role of APC in this process.

Acknowledgments—We thank Patrice Morin, Hans Clevers, and Keith Orford for the WT APC expression plasmid, LEF reporters, and S37A β-catenin construct, respectively.

[2] V. Easwaran and S. Byers, unpublished observations.

REFERENCES

1. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., and Robertson, M. (1991) *Cell* **66,** 589–600
2. Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., and Robertson, M. (1991) *Cell* **66,** 601–613
3. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., and Hedge, P. (1991) *Science* **253,** 665–669
4. Miki, Y., Nishisho, I., Miyoshi, Y., Horii, A., Ando, H., Nakajima, T., Utsunomiya, J., and Nakamura, Y. (1991) *Jpn. J. Cancer Res.* **82,** 1003–1007
5. Miyoshi, Y., Ando, H., Nagase, H., Nishisho, I., Horii, A., Miki, Y., Mori, T., Utsunomiya, J., Baba, S., and Petersen, G. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4452–4456
6. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992) *Hum. Mol. Genet.* **1,** 229–233
7. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., and Fukayama, M. (1994) *Cancer Res.* **54,** 3011–3020
8. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Willson, J. K., Paraskeva, C., Petersen, G. M., Hamilton, S. R., and Vogelstein, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2846–2850
9. Polakis, P. (1995) *Curr. Opin. Genet. Dev.* **5,** 66–71
10. Su, L., Vogelstein, B., and Kinzler, K. W. (1993) *Science* **262,** 1734–1737
11. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3046–3050
12. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. (1993) *Science* **262,** 1731–1734
13. Rubinfeld, B., Albert, I., Porfiri, E., Munemitsu, S., and Polakis, P. (1997) *Cancer Res.* **57,** 4624–4630
14. Gumbiner, B. (1997) *Curr. Opin. Cell Biol.* **7,** 634–640
15. Cadigan, K. M., and Nusse, R. (1997) *Genes Dev.* **11,** 3286–3305
16. Peifer, M., Sweeton, D., Casey, M., and Wieschaus, E. (1994) *Development* **120,** 369–380
17. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996) *Mol. Cell. Biol.* **16,** 2128–2134
18. Cook, D., Fry, M. J., Hughes, K., Sumathipala, R., Woodgett, J. R., and Dale, T. C. (1996) *EMBO J.* **15,** 4526–4536
19. Clevers, H., and van de Wetering, M. (1997) *Trends Genet.* **13,** 485–489
20. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) *Science* **275,** 1784–1787
21. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) *Science* **275,** 1787–1790
22. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) *Science* **281,** 1509–1512
23. Bullions, L. C., and Levine, A. J. (1998) *Curr. Opin. Oncol.* **10,** 81–87
24. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) *EMBO J.* **16,** 3797–3804
25. Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M., and Byers, S. W. (1997) *J. Biol. Chem.* **272,** 24735–24738
26. Polakis, P. (1997) *Biochim. Biophys. Acta* **1332,** F127–F147
27. Ciechanover, A. (1994) *Cell* **79,** 13–21
28. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
29. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) *Cell* **78,** 761–771
30. Lee, D. H., and Goldberg, A. L. (1998) *Trends Cell Biol.* **8,** 397–403
31. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Louriero, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997) *Cell* **88,** 789–799
32. Yang, L., Kim, H. T., Munoz-Medellin, D., Reddy, P., and Brown, P. H. (1997) *Cancer Res.* **57,** 4652–4661
33. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997) *Science* **275,** 1790–1792
34. Fukuchi, T., Sakamoto, M., Tsuda, H., Maruyama, K., Nozawa, S., and Hirohashi, S. (1998) *Cancer Res.* **58,** 3526–3528
35. Voeller, H. J., Truica, C. I., and Gelmann, E. P. (1998) *Cancer Res.* **58,** 2520–2523
36. Miyoshi, Y., Iwao, K., Nagasawa, Y., Aihara, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., and Nakamura, Y. (1998) *Cancer Res.* **58,** 2524–2527
37. Palacios, J., and Gamallo, C. (1998) *Cancer Res.* **58,** 1344–1347
38. Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M., and Bodmer, W. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10330–10334
39. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) *Nature* **396,** 590–594
40. Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B., and Polakis, P. (1994) *Cancer Res.* **54,** 3676–3681
41. Munemitsu, S., Albert, I., Rubinfeld, B., and Polakis, P. (1996) *Mol. Cell. Biol.* **16,** 4088–4094
42. Li, C., Bapat, B., and Alman, B. A. (1998) *Am. J. Pathol.* **153,** 709–714
43. Stambolic, V., Ruel, L., and Woodgett, J. R. (1996) *Curr. Biol.* **6,** 1664–1668
44. Kao, K. R., and Elinson, R. P. (1998) *Biol. Cell* **90,** 585–589
45. Klein, P. S., and Melton, D. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8455–8459
46. Hedgepeth, C. M., Conrad, L. J., Zhang, J., Huang, H. C., Lee, V. M., and Klein, P. S. (1997) *Dev. Biol.* **185,** 82–91
47. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., and Kikuchi, A. (1998) *J. Biol. Chem.* **273,** 10823–10826
48. Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998) *EMBO J.* **17,** 1371–1384
49. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996) *Science* **272,** 1023–1026
50. Yamamoto, H., Kishida, S., Uochi, T., Ikeda, S., Koyama, S., Asashima, M., and Kikuchi, A. (1998) *Mol. Cell. Biol.* **18,** 2867–2875
51. Nikolakaki, E., Coffer, P. J., Hemelsoet, R., Woodgett, J. R., and Defize, L. H. (1993) *Oncogene* **8,** 833–840
52. Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998) *Curr. Biol.* **8,** 573–581
53. Sakanaka, C., Weiss, J. B., and Williams, L. T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3020–3023
54. Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, K., and Akiyama, T. (1998) *Genes Cells* **3,** 395–403
55. Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998) *Science* **280,** 596–599
56. Jiang, J., and Struhl, G. (1998) *Nature* **391,** 493–496
57. Marikawa, Y., and Elinson, R. P. (1998) *Mech. Dev.* **77,** 75–80

Exogenous Expression of β-Catenin Regulates Contact Inhibition, Anchorage-independent Growth, Anoikis, and Radiation-induced Cell Cycle Arrest

Keith Orford, Caroline C. Orford, and Stephen W. Byers

The Lombardi Cancer Center and the Department of Cell Biology, Georgetown University School of Medicine, Washington, District of Columbia 20007

Abstract. β-Catenin is an important regulator of cell–cell adhesion and embryonic development that associates with and regulates the function of the LEF/TCF family of transcription factors. Mutations of β-catenin and the tumor suppressor gene, adenomatous polyposis coli, occur in human cancers, but it is not known if, and by what mechanism, increased β-catenin causes cellular transformation. This study demonstrates that modest overexpression of β-catenin in a normal epithelial cell results in cellular transformation. These cells form colonies in soft agar, survive in suspension, and continue to proliferate at high cell density and following γ-irradiation. Endogenous cytoplasmic β-catenin levels and signaling activity were also found to oscillate during the cell cycle. Taken together, these data demonstrate that β-catenin functions as an oncogene by promoting the G_1 to S phase transition and protecting cells from suspension-induced apoptosis (anoikis).

Key words: β-catenin • oncogene • cell cycle • anoikis • apoptosis

β-Catenin is a 92–97-kD protein associated with the intracellular tail of the intercellular adhesion molecule E-cadherin (Ozawa et al., 1989). Through this association, β-catenin plays an important role in strong cell–cell adhesion as it links E-cadherin (and other members of the cadherin family) to the actin cytoskeleton through the protein α-catenin (Hirano et al., 1992; Kemler, 1993). One mechanism by which cell–cell adhesion can be negatively regulated is via the phosphorylation of β-catenin on tyrosine residues (Behrens et al., 1993). There are some indications that this may be an important event in the transition from a benign tumor to an invasive, metastatic cancer (Sommers et al., 1994).

β-Catenin is also a regulator of embryogenesis, a role that was first suspected when it was shown to be the mammalian homolog of the *Drosophila* segment polarity gene *Armadillo* (Peifer et al., 1992). Further studies in *Drosophila* and *Xenopus* have revealed that β-catenin is a component of the highly conserved Wnt/Wingless signal transduction pathway that regulates body patterning in both species (Peifer, 1995; Gumbiner, 1997).

The membrane-associated and cytoplasmic pools of β-catenin have disparate activities: adhesion and signaling, respectively. The accumulation of cytoplasmic β-catenin drives its interaction with members of the LEF/TCF family of nuclear transcription factors that results in altered gene expression, which is the transduction of the Wnt/Wg signal (Clevers and van de Wetering, 1997). This accumulation of cytoplasmic β-catenin is regulated at the level of its degradation (Peifer et al., 1994; Peifer, 1995; Papkoff et al., 1996). In the absence of the Wnt/Wg signal, phosphorylation of specific serine residues on β-catenin leads to its ubiquitination and degradation, removing it from the cytoplasm (Orford et al., 1997). Mutations of these serine residues inhibit the ubiquitination of β-catenin, which causes it to accumulate and signal constitutively (Morin et al., 1997; Orford et al., 1997).

Along with its position in a growth factor signaling pathway, the demonstration of an interaction between β-catenin and the product of the tumor suppressor gene, adenomatous polyposis coli (APC)[1], suggests that it is involved in oncogenesis (Rubinfeld et al., 1993; Peifer, 1997). Tumor cell lines with a loss of one copy of APC, and harboring mutations in the other allele, have high lev-

Address correspondence to Dr. Stephen Byers, E415 The Research Building, GUMC, 3970 Reservoir Road N.W., Washington, DC 20007. Tel.: (202) 687-1813. Fax: (202) 687-7505. E-mail: byerss@gunet.georgetown.edu

1. *Abbreviations used in this paper:* APC, adenomatous polyposis coli; CON, control; EMT, epithelial to mesenchymal transition; FAK, focal adhesion kinase; HA, hemagglutinin epitope; ILK, integrin-linked kinase; PKC, protein kinase C; S37A, S37A mutant β-catenin plasmid; WT, wild-type β-catenin plasmid.

els of cytoplasmic (signaling) β-catenin, which is markedly reduced when functional APC is reintroduced (Munemitsu et al., 1995). Importantly, all mutant forms of APC found in human cancers are unable to reduce β-catenin levels in these cells. The importance of elevated β-catenin in human cancer was further substantiated when mutations in the β-catenin gene were described in colon cancer and melanoma cell lines (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). At least one of these mutations results in a more stable form of the protein.

A retroviral insertion screen for oncogenes using the NIH-3T3 cell line also implicated β-catenin as a possible oncogene, as the insertion of the retrovirus resulted in the expression of a β-catenin protein that lacked the NH_2 terminus (Whitehead et al., 1995). In contrast, overexpression of a stabilized form of β-catenin is unable to mimic the morphological effects of Wnt-1 in fibroblasts (Young et al., 1998).

Although much is now known about this signaling system, the actual cellular processes in which β-catenin plays a regulatory role is unclear. As described above, it regulates cadherin-mediated cell–cell adhesion. Although it appears to regulate gene expression, few target genes have been demonstrated. Based on its relationship with Wnt and APC, it is possible that β-catenin may positively regulate cellular proliferation or inhibit apoptosis. It is also tempting to speculate that the adhesive and the putative oncogenic functions of β-catenin are related and that it may be, at least in part, the mechanistic link between cell–cell adhesion, contact inhibition, and/or apoptosis. However, no studies have directly tested the hypothesis that β-catenin is actually oncogenic.

This report utilizes the MDCK cell line to determine the impact of overexpressing wild-type or a stabilized mutant form of β-catenin in nontransformed epithelial cells. The data demonstrate that β-catenin alters cell cycle progression and confers enhanced growth in soft agar, a surrogate marker for tumorigenicity. In addition, β-catenin confers resistance to suspension-mediated apoptosis (anoikis), radiation-induced cell cycle arrest, and allows cells to continue cycling when cultured at confluence. In short, β-catenin functions as an oncogene in the MDCK normal epithelial cell line.

Materials and Methods

Cells, Plasmids, and Stable Transfections

MDCK cells are a canine kidney-derived nontransformed epithelial cell line that are maintained in DME (GIBCO BRL), supplemented with 5% FBS. A1N4 cells are a human mammary nontransformed epithelial cell line that are grown in IMEM, supplemented with 0.5% FBS, 0.5% hydrocortisone, 5 μg/ml insulin, and 10 ng/ml EGF (Stampfer and Bartley, 1988). These cells synchronize in G_0 in the absence of EGF. The wild-type (WT) and S37A mutant (S37A) β-catenin plasmids were described previously (Orford et al., 1997). The bacterial chloramphenicol acetyltransferase gene driven by the CMV promoter of the pcDNA 3 plasmid (Invitrogen Corp.) served as the negative control (CON). For stable transfections, 800,000 MDCK cells were plated per 100-mm tissue culture plate. The next day, 15 μg of the various plasmids were transfected using the lipofectamine PLUS method (GIBCO BRL): 32 μl lipofectamine and 45 μl PLUS reagent. All of the plasmids included the neomycin-resistance cassette for selection. 48 h later, the cells were split 1:20 and cultured for 2 wk in the presence of 500 μg/ml of Geneticin (GIBCO BRL). An approximately equal number of colonies grew up for each transfected plasmid. For each transfection, all of the colonies were trypsinized and combined to give stable cell pools.

Immunoblotting

Whole cell and cytoplasmic lysates were made and immunoblotting performed as described previously (Orford et al., 1997).

Immunofluorescence

Cells were grown to confluence in 4-well BIOCOAT chamber slides (Falcon Plastics). Cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Cells were then permeabilized in 0.2% Triton X-100, 4% paraformaldehyde in PBS for 10 min. After washing in PBS, cells were blocked in 3% ovalbumin for 1 h. The chambers were incubated with primary antibodies overnight at 4°C. After washing in PBS five times for 5 min each, fluorescein- or Texas red-conjugated secondary antibodies were added for 1 h. Primary and secondary antibodies were diluted in 6% normal goat serum. After removal of the secondary antibody, the chambers were washed five times for 5 min in PBS, and the chambers removed. The cells were mounted with Vectashield (Vector Labs, Inc.).

Antibodies

The anti–β-catenin (C19220) and anti-p27 (K25020) mAbs were from Transduction Laboratories. The antihemagglutinin mAb (HA-11) was purchased from Berkeley Antibody Co., Inc. A second high affinity anti-HA mAb was purchased from Boehringer Mannheim Corp. (#186723). The anti–E-cadherin (SHE78-7) mAb was purchased from Zymed Labs, Inc. Peroxidase- and fluorescein-labeled secondary antibodies were purchased from Kirkegaard and Perry Laboratories, Inc. The Texas red-labeled secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc.

β-Catenin–LEF/TCF Signaling Assays

In 12-well dishes, cells were transfected with 0.5 μg of the TOPFLASH LEF/TCF reporter plasmid (van de Wetering et al., 1997) and 0.005 μg of the constitutively expressed Renilla luciferase, as a normalization control. As a negative control, cells were transfected with the FOPFLASH reporter plasmid in which the LEF/TCF binding sites have been mutated. The cells were lysed and assayed for Firefly and Renilla luciferase activities using the STOP & GLO assay (Promega Corp.). All results are normalized to the Renilla luciferase activity.

Soft Agar Growth Assay

For each cell pool, 150,000 cells were suspended in 3 ml DME + 5% FBS, and warmed to 37°C. 300 μl of a prewarmed (52°C) 3% agarose/PBS solution was mixed with the cell suspension and then layered into 3 wells of a 6-well plate (1 ml/well), which were previously coated with 1 ml of 0.6% agarose in DME. The agar was allowed to solidify at room temperature for 20 min before 3 ml of growth medium was added to each well. The medium was changed every three days. After 14 d, the colonies were counted by an Omnicon 3600 Colony Counter and photographed.

Growth Curves

To have an equal number of cells plated at the first time point, 10,000 CON, and 5,000 WT and S37A cells were plated per well of 12-well plates. At each time point, the cells were washed once in PBS and trypsinized in 1 ml trypsin/versene (GIBCO BRL). The single cell suspension was counted on a Coulter Counter set at 10 μm min with 20-μm maximum diameter. Each data point was performed in triplicate.

Plating Efficiency Assay

For each cell pool, 100 cells were plated onto each of three 100-mm tissue culture dishes in DME + 5% FBS. 4 d after plating, the colonies were photographed at 400×. After 8 d, the cells were washed with PBS, stained with crystal violet, and washed with water. The colonies were counted and then photographed. The plating efficiency is the mean number of colonies per dish/100 cells plated per dish.

Quantification of Cell Shedding

Cells were cultured in 6-well plates 3 d after confluence. The cells were

washed twice in PBS and 2 ml of fresh medium was added to each well. 24 h later, the shed cells were removed with medium and counted on a Coulter Counter, as described.

Cell Cycle Analyses

Two flow cytometric assays were used.

Vindelov Method. Cells were washed in PBS and trypsinized. Cells were washed in PBS and pelleted. After removing the wash buffer, the pellet was vortexed and resuspended in 0.1 ml of citrate/DMSO buffer (250 mM sucrose, 40 mM $Na_3C_6H_5O_7$ $2H_2O$, 5% DMSO, pH 7.60). The pellets were then frozen at −80°C. The cells were then processed as in Vindelov et al. (1983).

Ethanol Fixation Method. Cells were washed once in PBS and trypsinized. Trypsinized cells were pelleted at 1000 *g* and washed in 5 ml cold PBS. After a second centrifugation, the cells were resuspended in 0.5 ml cold PBS and fixed by dripping in 1.5 ml cold 100% ethanol, while slowly vortexing the cell suspension. After at least 1 h at 4°C, the cells were stained with propidium iodide and DNA content was measured by flow cytometry. The ethanol fixation method was also used for the flow cytometric analysis of apoptosis.

Cell Synchronization Experiments

β-Catenin Protein Level. A1N4 cells were plated in 100-mm tissue culture dishes and grown overnight to ~40% confluency. The cells were washed three times in PBS and then maintained in the absence of EGF for 46–50 h. This synchronized >95% of the cells in the G_0/G_1 phase of the cell cycle. To stimulate reentry into the cell cycle, EGF-containing medium was added back to the cells. Parallel dishes were analyzed at each time point for β-catenin protein (whole cell or the cytoplasmic pool) and for the cell cycle distribution.

β-Catenin–LEF/TCF Signaling. 50,000 A1N4 cells were plated per well of 12-well dishes and transfected with 1 μg of the TOPFLASH reporter plasmid and 0.01 μg of the Renilla control plasmid by the calcium phosphate method. The cells were then synchronized by EGF starvation (G_0/G_1) or 1 μM nocodazole (G_2/M), or treated with the proteosomal inhibitor ALLN, which stabilizes β-catenin. The cells were collected and the luciferase measurements were made as described.

Anoikis Assays

Confluent cells were trypsinized into a single cell suspension. 700,000 cells were plated in 150-mm tissue culture dishes coated with 0.8% agarose, to which they could not attach. At the various time points, the cells were collected, washed in PBS, and any cell aggregates were dispersed by trypsinization. Cells were then analyzed for apoptosis using three separate assays.

DNA/Flow Cytometry. Samples were analyzed by flow cytometry (see Cell Cycle Analyses, Ethanol Fixation). In this analysis, the hypodiploid peak constituted the apoptotic population.

AnnexinV Labeling. Samples were stained with fluorescein-labeled AnnexinV and propidium iodide (Trevigen) according to the manufacturer's protocol, and analyzed by flow cytometry. The two AnnexinV positive quadrants of the analysis were taken as the apoptotic fraction.

Hoechst Staining. Cells were fixed in 10% formalin for 10 min and stained with Hoechst #33258 (25 μg/ml in PBS) for 10 min at room temperature in the dark. Cells were placed on a glass slide and analyzed by fluorescence microscopy.

γ-Irradiation

750,000 CON, and 500,000 WT and S37A cells were plated in T75 tissue culture dishes. 26 h later, the flasks were exposed to 5 Gy of γ-irradiation. Another group of flasks received a mock irradiation (0 Gy). At 8 and 24 h after irradiation, the cells were trypsinized and their cell cycle profile was determined.

Results

Expression of β-Catenin Transgenes in MDCK Cells

To investigate the effects of β-catenin on normal cellular function, MDCK cells were stably transfected with constitutively expressed β-catenin transgenes that have been engineered to contain a COOH-terminal HA tag. In addition to WT β-catenin, a construct harboring a previously described serine to alanine point mutation at residue 37 (S37A) was used, which encodes for a β-catenin protein largely resistant to ubiquitination (Orford et al., 1997). The cells used are pooled stable transfectants; that is, after selection with G418, all of the drug resistant colonies resulting from each transfection were combined. These will be referred to as cell pools. As a negative control, a cell pool expressing the bacterial chloramphenicol acetyl transferase gene was generated (CON). Stable cell pools were generated to avoid the phenotypic artifacts that can result from the selection and propagation of individual clones derived from single transfected cells. We found that MDCK cells are especially prone to clonal morphological variation.

When examined by immunoblotting, expression of the HA tag was detectable only in the cell pool expressing the more stable S37A mutant (Fig. 1, B–E). We believe that epitope inaccessibility and antibody insensitivity result in the poor detection of the HA-tagged β-catenin and, consequently, the HA tag was undetectable by immunoblotting in untreated WT cells. To demonstrate that the WT cells were capable of expressing HA-tagged β-catenin, all three cell pools were treated with the histone deacetylatase inhibitor sodium butyrate to nonspecifically increase gene expression. This treatment resulted in clearly detectable expression in the WT cells and very high expression in the S37A cells, whereas the CON cells lacked expression under both conditions. Sodium butyrate treatment was not used in any other experiments in this study. In untreated cells, a similar pattern was seen by immunofluorescence microscopy. Using an antibody specific for the HA tag and a fluorescein-labeled secondary antibody, staining was detectable in the S37A cell pool (Fig. 1 E), but was difficult to detect in the WT cells (data not shown). To demonstrate the HA tag in the WT cells, a high affinity anti-HA antibody (Boehringer Mannheim) and a Texas red-conjugated secondary antibody was used to increase the sensitivity of the assay. Under these conditions, expression of the HA-tagged protein was clearly demonstrable in most of the WT cells (Fig. 1 D), even in the absence of butyrate, whereas expression was not evident in the CON cells (Fig. 1 C). A β-catenin specific antibody revealed a normal staining pattern in all three cell pools (Fig. 1, F–H).

Whole cell lysates do not exhibit any significant increase in total β-catenin levels (data not shown) because MDCK cells express a large amount of endogenous β-catenin, most of which is complexed with E-cadherin at the cell membrane. However, it is the cytoplasmic pool that is involved in β-catenin signaling and an increase in this pool was evident in both WT and S37A expressing cells, as compared with the CON cell pool (Fig. 1 A).

To confirm that β-catenin was being functionally overexpressed in both the WT and S37A cell pools, LEF/TCF-dependent nuclear signaling was measured using the TOPFLASH reporter construct (van de Wetering et al., 1997). This reporter consists of four consensus LEF/TCF binding sites placed upstream of the cFos minimal promoter. As a negative control, a similar reporter construct (FOPFLASH), in which the LEF/TCF binding sites have been

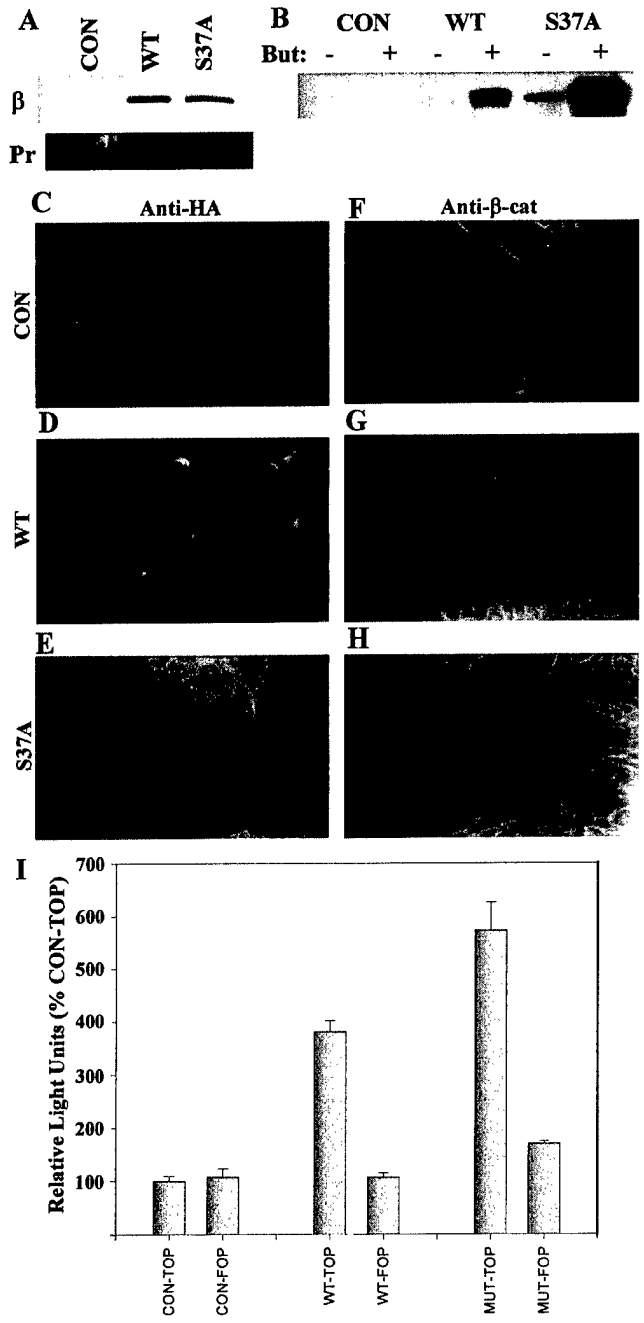


Figure 1. Expression of transgenes in MDCK stable cell pools. A, Equal protein from cytoplasmic extracts of CON, WT, and S37A cell pools was immunoblotted with an anti–β-catenin antibody. Amino black staining of nitrocellulose membrane demonstrates equal protein loading. B, Expression of HA-tagged β-catenin was determined by immunoblotting equal protein from whole cell lysates of the three cell pools cultured with and without sodium butyrate (But; to enhance gene expression) with anti-HA antibody (HA-11; BabCo). C–E, HA-tagged β-catenin can be detected in the WT (D) and S37A (E) cell pools by immunofluorescence. The HA-tag is absent in the CON cell pool (C). F–H, Expression of β-catenin in the same cell pools. I, β-catenin signaling activity was determined with the TOPFLASH LEF/TCF-responsive reporter construct. β-Catenin–LEF/TCF signaling is elevated above CON in both the WT and S37A cells. The negative control FOPFLASH reporter is essentially unaffected by β-catenin transfection.

mutated, was used. Even though the HA tag was not easily detected in the untreated WT cell pool, LEF/TCF signaling is elevated well above the control (Fig. 1 I, CON) in both the WT and S37A cell pools.

β-Catenin Overexpression Alters Cell Morphology

Overexpression of β-catenin in MDCK cells previously was shown to alter cell morphology. The stable cell pools used in this report have essentially the same morphology as the MDCKs expressing an inducible form of NH_2 terminally truncated β-catenin (Barth et al., 1997). The WT and S37A cell pools are less efficient at forming tight colonies of cells, as compared with CON cells (Fig. 2). In addition, the cells along the edges of the WT and S37A colonies tend to extend projections more readily, giving them a more mesenchymal morphology. The morphology of these cell pools also varied at high density. In contrast to their appearance at lower density, the WT and S37A cells appeared to be more tightly adherent to each other (data not shown). This is supported by the fact that these cells are significantly slower to round up when trypsinized during normal cell passaging. To confirm that expression of the β-catenin transgenes did not prevent strong intercellular adhesion, the ability of the WT and S37A cells transepithelial resistance was measured in the presence and absence of Ca^{2+}. Both the WT and S37A cells formed a strong barrier in the presence of Ca^{2+} (>1,000 ohms/chamber) that was completely diminished in the absence of Ca^{2+}. These results are consistent with what is seen in normal epithelial cell lines and confirms strong cadherin-mediated adhesion.

β-Catenin Stimulates Cell Proliferation

To characterize the distribution of these cells in the cell cycle, DNA/flow cytometry analysis was performed on these cells during exponential growth phase. Both of the β-catenin overexpressing cell pools had a reduced proportion of G_0/G_1 cells and an increased proportion of S and G_2 cells, as compared with the control cells (Fig. 3 A). This suggests that either a greater proportion of the WT and S37A cells are cycling or the G_1 phase of the cycle is shorter in duration than it is in the CON cells.

Growth curves demonstrated a significant difference between the β-catenin overexpressing cells (WT and S37A) and the CON cells (Fig. 3 B). The curves depicting the growth of the WT and S37A cell lines diverged from that of the CON cells, demonstrating that the alterations in cell cycle distribution resulted in increased growth. Also, overexpression of β-catenin increased saturation density of these cells (Fig. 3 B, inset). Together with the demonstration that the WT and S37A cells proliferate more rapidly at confluence (Fig. 4), it is clear that β-catenin overexpression significantly diminishes the property of contact inhibition of growth.

Interestingly, in every replication of this experiment, the number of cells in the WT and S37A wells was elevated (up to 50%) above the CON cells at the first time point of the growth curve. To determine if a difference in plating efficiency might explain the discrepancy in the cell number on the first day of the growth curves, 100 cells were plated per 100-mm tissue culture dish in three dishes for each cell pool. The colony count provides a rough estimate of the

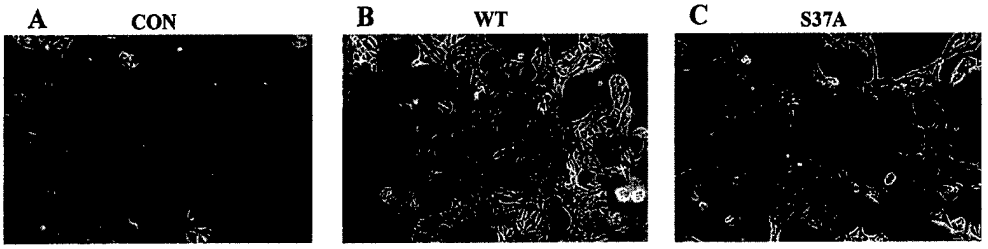


Figure 2. Exogenous β-catenin expression alters morphology of MDCK cells. Phase-contrast photographs of CON (A), WT (B), and S37A (C) cell pools demonstrate the effect of β-catenin overexpression on MDCK cell pools. β-Catenin-expressing cells show a more spindly, mesenchymal, less cell–cell adhesive morphology compared with the control cells.

plating efficiency of the cells. This experiment revealed a small (but not statistically significant) difference in plating efficiency that may contribute to the consistent differences in cell number, but does not explain them entirely (Fig. 3 C). We believe that the combination of increased plating efficiency and elevated proliferation rate account for the differences seen at the first time point.

An obvious increase in the rate of colony growth in the β-catenin overexpressing cells was more dramatic. The colonies from the WT and S37A cells were many fold larger than those from the CON cells. The morphology of these clones provides one explanation for the difference in colony size (Fig. 3, D, E, and F). Whereas the CON cells formed tightly adhesive, epithelioid colonies (Fig. 3 G), the WT and S37A cells formed a large number of colonies containing a more scattered, mesenchymal phenotype (Fig. 3 H). The morphological changes suggest that enhanced motility may contribute to this dramatic increase in colony size, but this is speculative. Also, the reduced adhesiveness in the WT and S37A cells may promote large colony formation by avoiding the contact inhibitory effect of tight cell–cell adhesion. In addition, other data suggest that the WT and S37A cells have an increased proliferative rate, even in the presence of strong intercellular adhesion (Fig. 4).

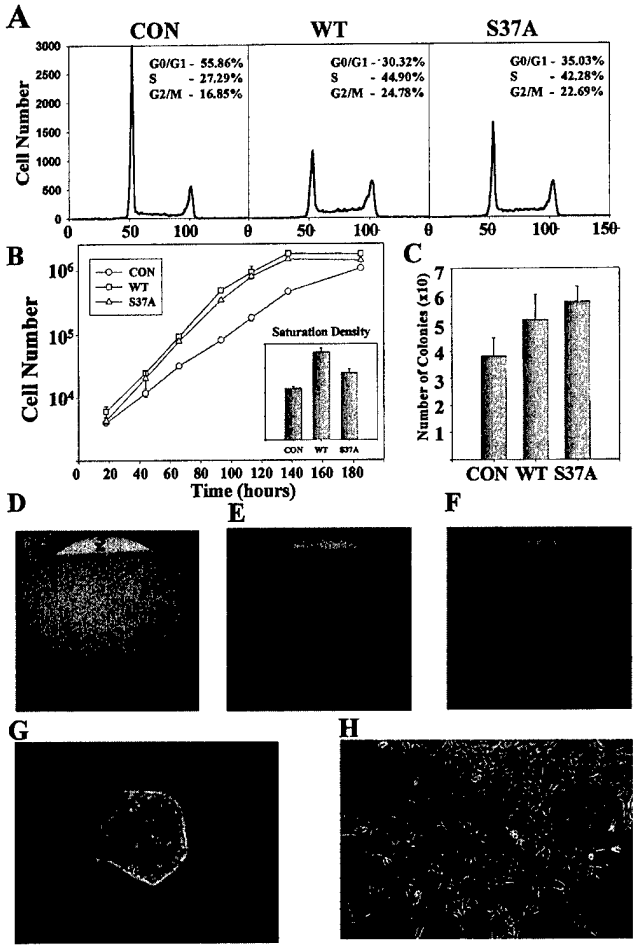


Figure 3. β-Catenin overexpression alters proliferation, plating efficiency, and colony morphology. A, DNA/flow cytometric analysis of the three cell pools during exponential growth demonstrates that the WT and S37A cell pools have a significantly lower percentage of cells in the G_0/G_1 phase of the cycle and a higher percentage in both S and G_2 phases of the cell cycle. B, Growth curves reveal that WT and S37A cells proliferate more rapidly than CON cells. To have approximately equal numbers of cells at time 0, 10,000 CON, 5,000 WT, and 5,000 S37A cells were plated per well in 12-well tissue culture plates. Each time point was done in triplicate. Graphing and SD calculations were performed with Sigmaplot. Error bars are hidden by symbols at several time points. Inset, saturation density of the three cell pools. Cells were counted at absolute confluence in 12-well plates. Each measurement is the mean of the cell counts from at least six wells. Graphing and SD calculation was performed with Sigmaplot. C–H, Plating efficiency assay reveals changes in colony morphology. 100 cells from each of the cell pools were plated in 100-mm dishes. After eight days, the colonies were stained with crystal violet, counted, and photographed. C, Number of colonies counted for each of the three cell pools. D–F, Photographs of crystal violet stained CON (D), WT (E), and S37A (F) colonies. G and H, Phase-contrast photographs of representative colonies from the CON (G) and WT (H) cell pools at four days. S37A colonies looked identical to the WT colony pictured. All experiments were performed at least three times with consistent and repeatable results.

β-Catenin Promotes Proliferation at High Cell Density

The reduction in proliferative rate that nontransformed cells experience at high cell density has been termed contact inhibition of growth. Although this is a widely recognized phenomenon, the signaling mechanisms involved remain unknown. To address this, the MDCK cell pools

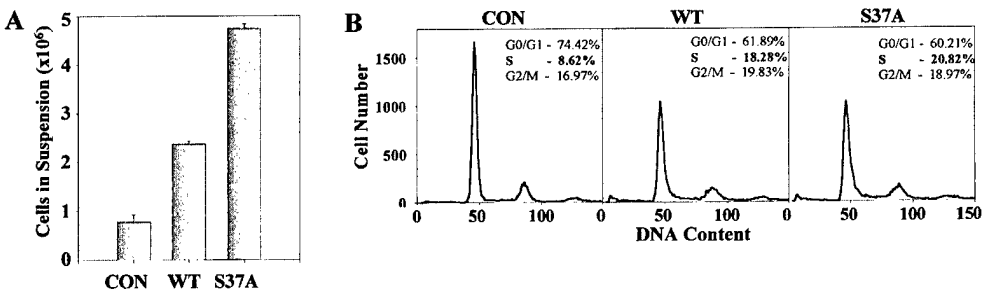


Figure 4. β-Catenin overexpression promotes proliferation at high cell density. A, Cells were cultured three days after confluence. After washing, cell shedding was measured over a 24 h period by counting the number of cells suspended in the medium. Graphing and SD calculation were performed using Sigmaplot. B, Cell cycle profiles of cells grown three days after confluence. After cells were washed twice with PBS, cell cycle analysis was performed on the adherent cells. The S phase percentage is increased 2–2.5-fold in WT and S37A cells relative to CON. All experiments were performed at least three times with consistent and repeatable results.

were grown to confluence and cell cycle parameters were monitored. Pilot experiments revealed that the WT and S37A cells shed more cells into the medium than CON cells. To quantify this effect, cells that were two to three days after confluence were washed twice with PBS, and fresh medium was added. The medium was collected from the wells on the next day and the suspended cells were counted. The number of shed cells was markedly elevated in the WT and S37A cells, as compared with the CON cells (Fig. 4 A). In these experiments, shedding of the S37A cell pool was consistently higher than in the WT cell pool.

The hypothesis that a higher proliferative rate was responsible for the difference in cell shedding was tested by performing cell cycle analysis of these cells grown three days after confluence. This analysis demonstrated that the WT and S37A cells had a higher proportion of S phase and G_2 phase, and a lower percentage of G_0/G_1 phase, as compared with the CON cells (Fig. 4 B). This cell cycle profile is precisely what would be expected if the WT and S37A cells were proliferating more rapidly than the CON cells, and is consistent with other experiments in which the G_1/S checkpoint control regulates contact inhibition (Dietrich et al., 1997; Kato et al., 1997). Presumably, in the absence of additional space to attach to the culture dish, the newly formed cells are shed into the medium.

β-Catenin Attenuates the Radiation-induced G_1/S Cell Cycle Block

One important aspect of cell cycle regulation is cell cycle blockade after DNA damage. These blocks, which occur at the G_1/S and G_2/M transitions, presumably allow the cell to repair its DNA before the damage-induced errors become permanent (Weinert, 1998). We postulated that β-catenin overexpression might alter the DNA damage-induced late G_1 block of the cell cycle in the MDCK cells. The three cell pools were γ-irradiated with 0 or 5 Gy. Eight hours after irradiation, all of the cell pools show some G_1/S and G_2/M cell cycle blockade (Fig. 5). However, while CON had very few S-phase cells (5.96%), the WT and S37A cells retained a significant number of cells in S phase (15.26 and 14.99%). 24 h after irradiation, 25.2 and 21.4% of the WT and S37A cells, respectively, were in S phase, compared with 0.77% of CON cells. These data demonstrate that the radiation-induced G_1/S block is strongly attenuated by the overexpression of β-catenin and indicates that elevated β-catenin might lead to the accumulation of DNA damage and increased incidence of other mutations.

β-Catenin Expression Fluctuates throughout the Cell Cycle

The previously described block of G_1/S progression by APC in normal cells points to a role of endogenous β-catenin in the regulation of cell cycle progression in nontransformed cells (Baeg et al., 1995). Together, with our demonstration that even the modest elevations of β-catenin described in this study can regulate cell cycle progression,

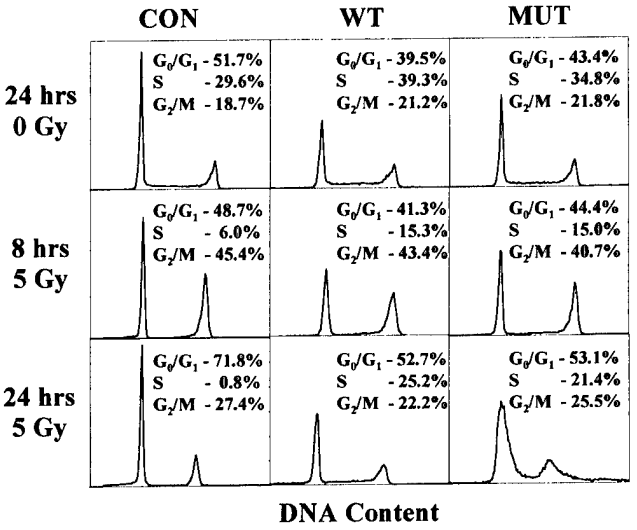


Figure 5. β-Catenin overexpression attenuates the γ-irradiation G_1 cell cycle block. Cells were γ-irradiated with 0 or 5 Gy. 8 and 24 h later, the cell cycle distribution was determined. The unirradiated cells all had a similar profile with the characteristic differences in G_0/G_1 and S phases (see Fig. 3). The S phase population of CON cells is significantly reduced at 8 h and absent at 24 h. At 24 h, the CON cells were blocked entirely in the G_0/G_1 or G_2 phases of the cell cycle. A slight decline in the proportion of WT and S37A cells in S phase occurs at 8 h after irradiation, but this is much less than that which occurs in CON cells. In contrast to CON cells, at 24 h the S phase proportions of the WT and S37A cells have partially recovered. All experiments were performed at least three times with consistent and repeatable results.

this led us to investigate its level of expression throughout the cell cycle. Preliminary experiments were performed with parental MDCK cells that were partially synchronized in early G_1 by serum starvation. Parallel wells of cells were collected at various time points after release from G_0 by the addition of serum to make whole cell or cytoplasmic lysates for analysis of β-catenin protein levels. Although total β-catenin protein did not vary appreciably during the cell cycle, cytoplasmic β-catenin levels increased significantly from G_1 to S phase (data not shown). The increase began in late G_1 and continued through S phase. These pilot experiments led us to examine this phenomenon in the A1N4 cell line, which is easily synchronized in early G_1 by the removal of EGF from the growth medium. Like MDCK cells, cytoplasmic levels of β-catenin protein increased in late G_1 and continued to rise in S phase (Fig. 6 A), whereas total cell β-catenin did not vary (data not shown). Densimetric scanning revealed a 23-fold increase in cytoplasmic levels from early G_1/G_0 to S phase (Fig. 6 B). As a control, the blot was reprobed for cyclin dependent kinase inhibitor, p27 (Fig. 6 A). As expected, variations in p27 were inversely related to β-catenin. To determine if this oscillation in cytoplasmic β-catenin led to fluctuations in β-catenin–LEF/TCF signaling, A1N4 cells were assayed for TOPFLASH activity after being synchronized in G_1 phase or G_2/M phase of the cell cycle. The level of β-catenin–LEF/TCF signaling corresponded with the levels of cytoplasmic β-catenin measured by Western blotting (Fig. 6 C). The elevation in signaling at G_2/M was greater than that induced by treatment with the proteosomal inhibitor, ALLN. These data indicate that oscillations in β-catenin signaling may be involved in the normal regulation of cell cycle progression.

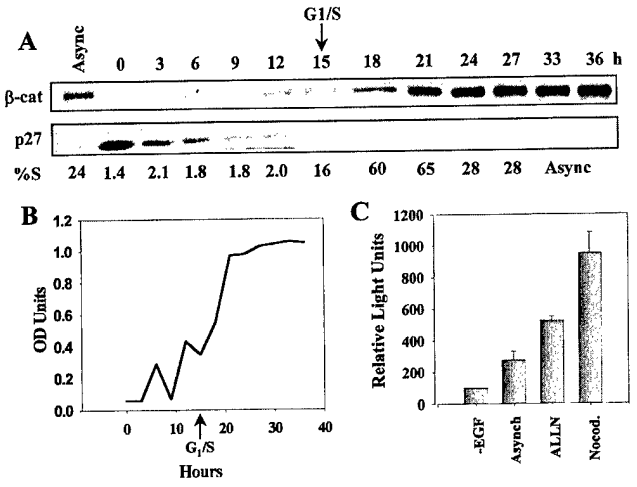


Figure 6. Cytoplasmic β-catenin oscillates during the cell cycle. A, A1N4 cells were synchronized in G_0/G_1 by EGF starvation. After releasing the cells into the cell cycle by the addition of EGF, cytoplasmic lysates were made every 3 h and assayed for β-catenin and p27 protein by immunoblotting. The distribution of cells in the cell cycle was determined at each time point by analyzing parallel cell cultures by flow cytometry. The percentage of S phase cells (%S) is provided. B, The level of expression was determined at each time point by densitometry and the results plotted against time after EGF addition. C, β-catenin–LEF/TCF signaling was measured in cells that were blocked in G_0/G_1 by EGF starvation (−EGF), growing asynchronously (Asynch), blocked near the S/G_2 transition by the proteosomal inhibitor ALLN, or blocked at G_2/M with nocodazole (Nocod). The results are expressed relative to the G_0/G_1 synchronized samples. Experiments represented in A and B were performed three times with consistent and repeatable results. Experiments represented in C were performed twice with consistent and repeatable results.

β-Catenin Promotes Colony Formation in Soft Agar

The ability of cells to proliferate in the absence of attachment to a solid substrate correlates well with the transformed, tumorigenic phenotype. To assess the oncogenic capacity of β-catenin in vitro, cells were suspended in 0.3% agar and allowed to grow for two weeks. The ability of the WT and S37A cells to form colonies in soft agar was clearly enhanced relative to the CON cells (Fig. 7, A–C). Although the CON cells do exhibit a background level of colony formation, expression of the β-catenin transgenes resulted in a 10–20-fold increase in the number of colonies and an obvious increase in colony size (Fig. 7 D). Multiple experiments did not demonstrate a significant difference between the WT and S37A cell pools. This is the first demonstration that full-length β-catenin, WT and S37A mutant, has transforming capacity.

β-Catenin Inhibits Anoikis

When nontransformed epithelial cells are deprived of attachment to an extracellular matrix for an extended period of time they undergo apoptosis (Frisch and Francis, 1994;

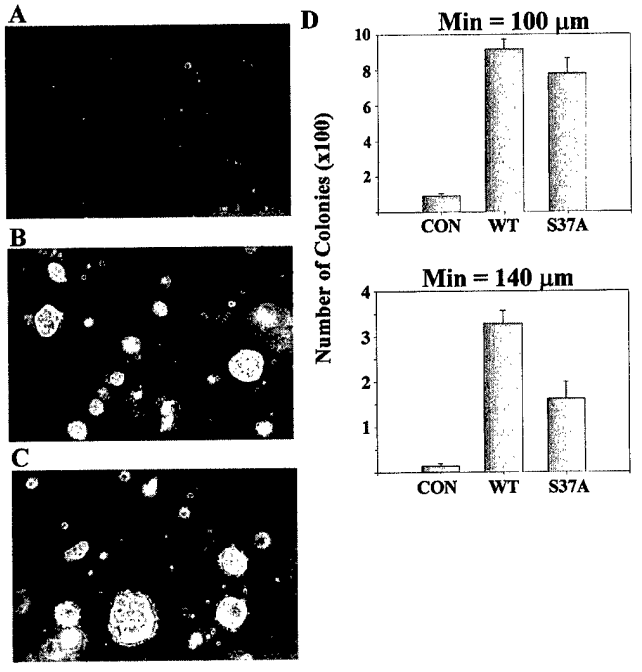


Figure 7. β-Catenin overexpression regulates soft agar colony formation. A–C, Phase-contrast photographs of colonies formed by the CON (A), WT (B), and S37A (C) cell pools after 14 d in soft agar. D, The number of colonies per 35-mm dish quantified by the Omnicon 3600 colony counter, using either 100 or 140 μm as the threshold for colony diameter. Experiments were repeated three times with consistent and repeatable results.

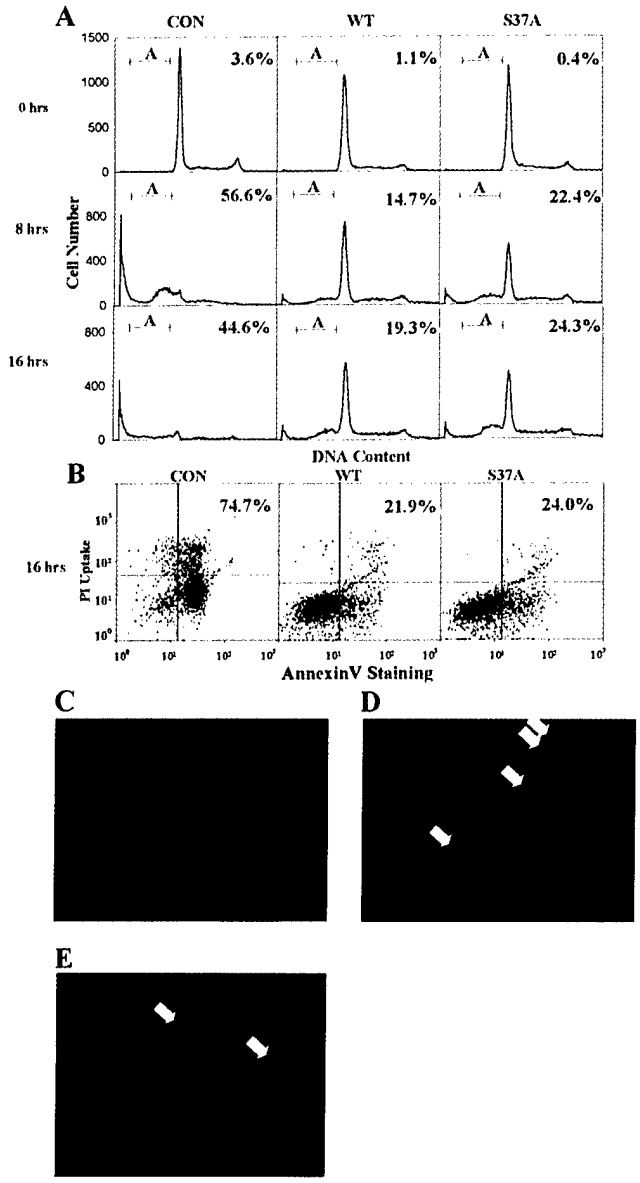


Figure 8. β-Catenin expression prevents anoikis. A, DNA/flow cytometric analysis of CON, WT, and S37A cells after incubation in suspension for 0, 8, or 16 h. The hypodiploid population corresponds to the apoptotic cells. The percentage in each panel represents the hypodiploid fraction. B, AnnexinV and propidium iodide staining of the same cells at 16 h also demonstrates a significant protection by β-catenin. C, Hoechst staining demonstrates nuclear morphology of CON cells before suspension. All nuclei look normal. WT and S37A cells looked similar. D, Hoechst staining of CON cells after 16 h in suspension. Most nuclei have a shrunken apoptotic morphology (arrows). E, S37A cells after 16 h in suspension. Most cells had the normal nuclear morphology, but a significant fraction (~25%) were shrunken apoptotic (arrows). Experiments represented in A and B were performed at least three times with consistent and repeatable results. Experiments represented in C–E were performed twice with consistent and repeatable results.

Frisch and Ruoslahti, 1997). This suspension-induced apoptosis has been termed anoikis. In the soft agar growth experiments, it appeared that most CON cells die when suspended in soft agar. However, the remaining cells did contribute to a background rate of colony formation. To investigate the possibility that β-catenin increases the colony-forming capacity of MDCK cells by preventing anoikis, cells were cultured on a cushion of 0.8% agar in normal growth medium, collected at eight hour intervals over a 24-h period, and assayed for apoptosis. Microscopic examination of the cells after 16- and 24-h incubations revealed that the majority of the WT and S37A cells were larger and more refractile to light than the CON cells (data not shown), suggesting that the CON cells were preferentially undergoing apoptosis. These preliminary results were confirmed by DNA/flow cytometry and AnnexinV staining of cells that had been kept in suspension for 0, 8, or 16 h (Fig. 8, A and B). Both methods showed that anoikis was significantly inhibited by β-catenin overexpression.

The results of further analysis of the flow cytometry and AnnexinV data for the percentage of hypodiploid and AnnexinV-positive cells, respectively, are compiled in Table I. The DNA/flow cytometry data revealed that the percentage of hypodiploid cells was markedly and consistently lower in the WT and S37A cells relative to the CON cells. However, these data significantly underestimate the percentage of apoptotic cells in the CON samples at the 16 h time point, as the disintegrating apoptotic cells were lost from the analysis. The AnnexinV assays appeared to retain these cells and probably give a more accurate estimate at 16 h.

As a third independent method of measuring apoptosis, nuclear morphology of cells before and after suspension was analyzed by Hoechst staining. In contrast to the nonsuspended cells, which all had normal nuclear morphology (Fig. 8 C), most of the suspended CON cells displayed characteristically shrunken apoptotic nuclei (Fig. 8 D). In contrast, the nuclei of the majority of WT and S37A cells displayed a normal morphology (Fig. 8 E). A fraction of the cells (~1/4) were apoptotic, which is consistent with the AnnexinV and flow cytometry results. Interestingly, a minority of CON cells were found to be associated with clumps of five or more cells. Most of these cells displayed normal nuclear morphology. This was a clear demonstration that cell–cell adhesion can prevent apoptosis induced by suspension, and this probably caused us to underestimate the percentage of apoptosis among the suspended CON cells by the AnnexinV and flow cytometric methodologies.

These data demonstrate that β-catenin overexpression may promote soft agar colony formation of MDCK cells by the promotion of cell cycle progression and the inhibition of anoikis.

Table I. β-Catenin Prevents Anoikis, as Measured by DNA/Flow Cytometry and AnnexinV Labeling

	Hypodiploid cells			AnnexinV positive		
	0 h	8 h	16 h	0 h	8 h	16 h
		%			%	
CON	3.6	**56.6**	44.6	1.7	50.4	**74.7**
WT	1.1	**14.7**	19.1	2.4	18.0	**21.9**
MUT	0.4	**22.4**	24.3	0.7	31.0	**24.0**

The percentage of apoptotic cells in the three cell pools after different periods of suspension, as measured by flow cytometry (hypodiploid) or AnnexinV labeling (AnnexinV positive). The percentages in **bold** demonstrate the most notable effects.

Discussion

It is suspected that the cadherin-associated protein β-catenin promotes the process of carcinogenesis (Peifer, 1997). The data that support this hypothesis include the following observations: it associates with and is downregulated by the tumor suppressor APC; it transduces (at least partly) the oncogenic Wnt growth factor signal to the nucleus; it is mutated in a significant number of human cancers; and, overexpression of an NH_2 terminally truncated form of β-catenin in the epidermis of transgenic mice produced well-differentiated hair tumors (Rubinfeld et al., 1993; Su et al., 1993; Cadigan and Nusse, 1997; Ilyas et al., 1997; Fukuchi et al., 1998; Gat et al., 1998; Miyoshi et al., 1998; Palacios and Gamallo, 1998; Voeller et al., 1998). However, no studies provide direct evidence for the transforming potential of full-length β-catenin. In addition, no investigations have addressed the question of which cellular processes β-catenin may regulate to effect cellular transformation.

β-Catenin Transforms the Epithelial MDCK Cell Line

This report characterizes phenotypic alterations that result from β-catenin overexpression in a nontransformed epithelial cell line. Effects are seen in the regulation of three important cellular activities/properties: proliferation, apoptosis, and morphology. It demonstrates that modest β-catenin overexpression significantly enhances the ability of these cells to proliferate, especially in situations that would normally inhibit the cell cycle at the G_1/S transition. Most striking is the demonstration that it promotes growth in soft agar, a phenotype closely correlated with tumorigenicity. Most nontransformed cells require adhesion through integrin receptors to extracellular matrix components to transit through the G_1 phase of the cell cycle (Mehta et al., 1986; Polyak et al., 1994). In addition, suspension of normal, attachment-dependent cells blocks them late in G_1 phase.

β-Catenin overexpression also resulted in increased proliferation of cells at high cell density. The mechanism by which high cell density inhibits proliferation is unknown, but also involves a block in late G_1. The presence of cell–cell adhesion, the reduction of cell-substrate adhesion, and the depletion of growth factors have all been implicated (Chen et al., 1997). β-Catenin's dual activities as a regulator of cadherin-mediated cell–cell adhesion and as the transducer of a mitogenic signal implicate it in this regulatory process. Both cadherin and α-catenin can inhibit β-catenin signaling in other experimental systems (Fagotto et al., 1996; Simcha et al., 1998). Together, with the results of the present study, these data support the hypothesis that cell–cell adhesion promotes the formation of cadherin/β-catenin/α-catenin complexes and that these complexes negatively regulate β-catenin signaling, which discourages cell cycle progression. However, the fact that proliferation is reduced at high cell density, as compared with sparsely plated cells, even in the WT and S37A cells, suggests that other mechanisms are also involved (for example, cell shape; Chen et al., 1997).

The cell cycle analyses and growth curves in this study demonstrate that β-catenin overexpression can significantly alter the proliferative rate of these cells. The distribution of the WT and S37A cells is weighted heavily toward S phase and away from G_1. When considered along with the other cell cycle data, it appears that β-catenin overexpression expedites the G_1/S transition in MDCK cells. The easing of the barrier to G_1/S transition manifests as a difference in cell growth on plastic, as growth curves of the β-catenin overexpressing cells diverged significantly from the control cells.

β-Catenin overexpression also has a notable effect on cell morphology. The MDCK cell line is a nontransformed epithelial line that has very strong intercellular adhesion and extends cell membrane extensions only to a limited degree. β-Catenin overexpression converts MDCKs into a more mesenchymal cell type (Barth et al., 1997; and the present study). At low density, cell–cell adhesion is reduced and the cells take on a more spindly, stretched shape. This change in morphology is reminiscent of an epithelial to mesenchymal transition (EMT; Huber et al., 1996). EMTs are developmentally important cellular conversions, especially during gastrulation, the point in development at which β-catenin knockout mouse embryos are aborted. Also, an EMT has been suggested to underlie the progression from benign tumor to metastatic carcinoma (Sommers et al., 1991; Birchmeier et al., 1996). Indeed, it previously has been suggested that β-catenin signaling may regulate this process (Sommers et al., 1994; Huber et al., 1996).

The absence of anoikis is another characteristic of transformed cells. The present study and others have shown that MDCK cells are very dependent on attachment to the extracellular matrix for survival (Frisch and Francis, 1994; Frisch et al., 1996a,b). After 16 h in suspension, the majority of CON cells were apoptotic, as measured by three independent methods. The expression of the WT and S37A β-catenin transgenes markedly retards this process, allowing ~75% of the single cells to survive. This is a vigorous inhibition of anoikis. Taken together, the proliferation, anoikis, and morphology data demonstrate that these cells are clearly transformed by β-catenin.

These in vitro results suggest that overexpression of full-length β-catenin should promote tumorigenesis in vivo. Two separate studies have demonstrated the effect of tissue-specific overexpression of an NH_2 terminally truncated form of β-catenin. Expression of the truncated form of β-catenin in the epidermis of transgenic mice by Gat et al. (1998) resulted in the formation of two types of hair follicle-related tumors. Taken together with the present study, these results strongly suggest that full-length forms of β-catenin are important mediators of oncogenesis in vivo. Interestingly, a study by Wong et al. (1998), in which an NH_2 terminally truncated form of β-catenin was overexpressed in the intestinal epithelium of transgenic mice, produced conflicting results. Proliferation of the intestinal epithelial cells in these animals was stimulated 1.5–3-fold, in accordance with the results of the present study. However, the elevated proliferation rate was balanced by an increase in apoptosis, the net result being no change in intestinal villus height. To explain the discrepancy between these results and our own, we suggest that β-catenin overexpression can protect cells only from certain apoptotic signals. It is possible that the compensatory mechanism by which the authors suggested that the transgenic mice might have maintained their cell census in the face of in-

creased proliferation is mediated through the stimulation of β-catenin–insensitive apoptosis. It is also possible that full-length β-catenin has signaling capacities that are lost when its NH_2 terminus is removed.

The results presented in the present study also differ from those published previously by Young et al. (1998). They reported that overexpression of the Wnt-1 growth factor transformed Rat-1 fibroblasts while expression of the S37A mutant form of β-catenin we described previously had no effect. Two differences between the two studies may explain the conflicting results. First, the morphological effects we describe may only be detectable in an epithelial cell type. Second, the studies of Young et al. (1998) were carried out without serum, whereas the present ones were done with serum. It is possible that Wnt-1 activates parallel signaling pathways (in addition to β-catenin signaling) that may circumvent the need for serum to stimulate proliferation. β-Catenin's position lower in the pathway may preclude the activation of such parallel pathways and, therefore, it is unable to stimulate proliferation of Rat-1 fibroblasts in the absence of serum.

β-Catenin Attenuates the Cell's Response to γ-Irradiation

The cell cycle blocks that characterize the response of cells to DNA damage are important for the maintenance of genomic integrity. To prevent the permanent incorporation of mutations induced by various DNA damaging stimuli, the cell cycle can pause at the G_1/S and the G_2/M transitions (Weinert, 1998). During these delays, the cell assesses the damage to its DNA and either repairs the damage or destroys itself. Premature reentry into the cell cycle may result in the accumulation of mutations to oncogenes and tumor suppressor genes, which would increase the likelihood of cellular transformation and cancer. The data from this study suggest that β-catenin overexpression may result in the premature reentry of cells into the cell cycle after γ-irradiation–induced DNA damage, and thereby promote the accumulation of oncogene mutations and carcinogenesis.

β-Catenin Overexpression Inhibits Anoikis

An association between apoptosis and the APC/β-catenin axis has been suggested previously. Reexpression of the APC gene in a tumor cell line that lacks WT APC resulted in the induction of apoptosis within 24 h (Morin et al., 1996). Since one of the functions of APC is to downregulate β-catenin, it is possible that β-catenin itself is a regulator of apoptosis. Our demonstration that β-catenin alone significantly protects cells from anoikis strongly implies that it can be a potent inhibitor of apoptosis. Also, during the process of apoptosis, caspase-3 can cleave β-catenin protein (Brancolini et al., 1997). One purpose of this cleavage may be to destroy the antiapoptotic β-catenin signal within the cell and thereby hasten the completion of the apoptotic process. The caspase-mediated cleavage of focal adhesion kinase (FAK) is thought to function in this manner (Wen et al., 1997).

It has been postulated that the induction of apoptosis by the loss of appropriate extracellular matrix attachment (i.e., anoikis) is a means of protecting the organism from improper cell growth (Frisch and Ruoslahti, 1997). Anoikis is prevented by integrin-mediated signaling. Several enzymes have been implicated as being downstream of integrins in this signal transduction pathway. These include FAK, phosphoinositide-3-kinase, protein kinase B/Akt, and integrin-linked kinase (ILK; Clark and Brugge, 1995; Giancotti, 1997; Wu et al., 1998). The present report suggests that β-catenin may also lie downstream of integrins. Several integrin-stimulated signaling pathways might lead to the induction of β-catenin signaling. One possible connection between integrins and β-catenin is the integrin-activated, antiapoptotic kinase PKB/Akt. PKB is known to inhibit the activity of glycogen synthase kinase 3-β, a serine kinase that functions directly to reduce β-catenin protein and signaling (Siegfried et al., 1992; Cook et al., 1996; Cadigan and Nusse, 1997). It is possible that the result of these two inhibitory interactions is that activation of PKB by integrin signaling functions to positively activate β-catenin signaling.

The data presented in this report describing the effects of β-catenin overexpression are similar to previous reports describing the effects of ILK (Novak et al., 1998; Wu et al., 1998). ILK is a 59-kD serine kinase that was first described as a β_1-integrin-associated kinase. ILK overexpression causes cells to undergo an EMT and promotes their growth in soft agar. This is associated with an increase in LEF-1 protein levels. As a result of increased LEF-1, β-catenin becomes completely localized to the nucleus and β-cat-LEF/TCF signaling increases significantly. In addition, loss of cell attachment to the underlying ECM was shown to result in a dramatic reduction in LEF protein. In a separate study, ILK directly phosphorylated and inhibited the activity of GSK-3β. This may constitute another mechanism by which integrin signaling may result in increased β-catenin–LEF/TCF signaling.

Anoikis results from the interruption of integrin-mediated signaling (Frisch and Ruoslahti, 1997). In addition to ILK, the integrin-associated nonreceptor tyrosine kinase FAK may also be involved in the transduction of these signals because FAK signaling suppresses p53-dependent apoptosis (Ilic et al., 1998). Ilic et al. (1998) also demonstrated that an atypical protein kinase C isoform (PKCλ/ι) is required for this p53-dependent apoptotic pathway, since inhibition with both chemical PKC inhibitors and a dominant–negative construct protect FAK-defective cells from apoptosis. Previously, we reported that an atypical PKC isoform was involved in regulating β-catenin degradation (Orford et al., 1997). Inhibiting atypical PKC activity using the same chemical PKC inhibitors used by Ilic et al. (1998) resulted in the inhibition of the ubiquitination and degradation of β-catenin. In addition, treatment of cells with these PKC inhibitors increases β-catenin–LEF/TCF signaling (unpublished results). Taken together with the present study, it is possible that the inhibition of PKCλ/ι or another atypical PKC may increase β-catenin stability and signaling, leading to the suppression of p53-mediated apoptosis (Fig. 9 A).

β-Catenin Oscillations during the Cell Cycle May Regulate Normal Cellular Proliferation

The c-myc promoter is also regulated by the APC/β-cate-

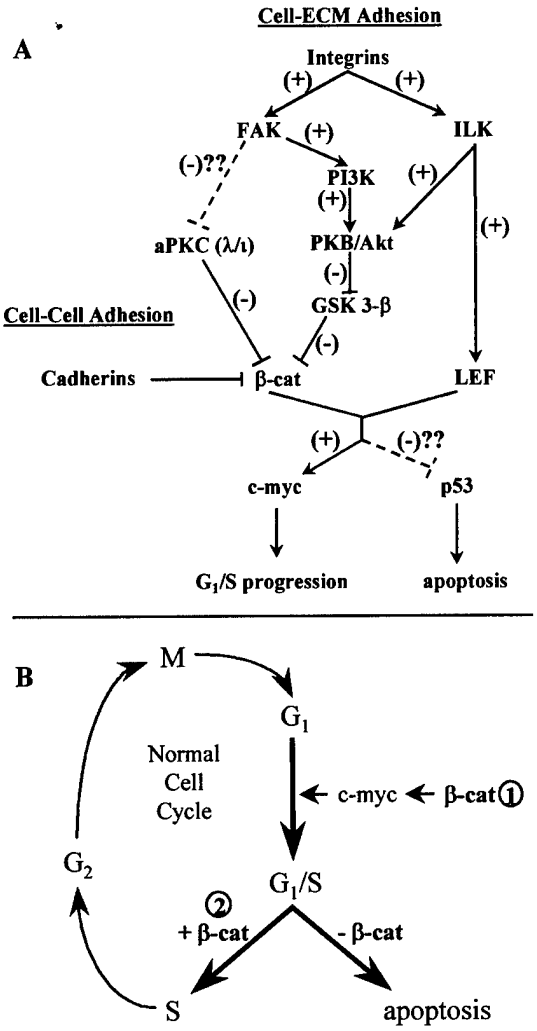


Figure 9. Hypothetical signaling pathways by which β-catenin might integrate cell adhesion, cell cycle, and apoptosis. A, The individual regulatory relationships depicted by unbroken arrows and blockades have been demonstrated in various published reports. However, these signaling pathways have never been demonstrated in their entirety within a single experimental system. The broken blockades are hypothetical regulatory events suggested in the present report. Integrin-activated FAK activity may regulate β-catenin signaling by two different pathways. In both cases, two sequential negative regulatory interactions downstream of FAK may result in the activation of β-catenin signaling. By a parallel pathway, ILK can regulate the activities of PKB and GSK-3β, as well as upregulate the expression of the transcription factor LEF-1. Together, β-catenin and LEF-1 might stimulate the G_1/S transition in the cell cycle (possibly via c-myc) and inhibit p53-mediated apoptosis. The inhibition of apoptosis may be through direct modulation of p53 action or through a parallel antiapoptotic pathway. The role of p53 in β-catenin–mediated signaling is speculative. B, β-catenin may regulate the cell cycle by two separate mechanisms: 1, β-catenin can stimulate the expression of c-myc, which is a strong stimulator of cell cycle progression; 2, the G_1/S transition represents an important decision-point for the cell. It is known that this transition requires the presence of survival factors. In their absence, the cell chooses apoptosis over proliferation. β-Catenin may regulate the G_1/S transition as a survival factor functioning to permit cell cycle progression by preventing apoptosis.

nin signaling pathway (He et al., 1998). The upregulation of c-myc by β-catenin may constitute one mechanistic link between β-catenin and tumor formation. c-myc is potent oncogene that regulates cell cycle progression. However, c-myc overexpression cannot induce cellular transformation on its own. In fact, when overexpressed alone, c-myc markedly increases the susceptibility of cells to apoptosis (Desbarats et al., 1996; Steiner et al., 1996; Thompson, 1998). To transform cells, c-myc requires an accompanying survival signal to prevent cells from undergoing apoptosis. Advancement through the G_1 phase of the cell cycle can result in either progression into S phase or apoptosis, depending on the presence or absence of certain survival signals, for example, IGF-1 (Evan et al., 1995). In addition to stimulating c-myc, β-catenin may transduce the requisite antiapoptotic signal that would permit cell cycle progression. The increase of cytoplasmic β-catenin protein before S phase during the cell cycle may serve this purpose in normal cells (Fig. 6). Additionally, β-catenin would protect against anoikis if overexpressed in epithelial cells.

Our data do not demonstrate any reproducible phenotypic difference between the WT and S37A expressing cells, except in the measurement of protein expression and in cell shedding at confluence. It is important to note that in both the WT and S37A cell pools, the level of cytoplasmic β-catenin protein and β-catenin–LEF/TCF signaling is elevated relative to the CON cells. This implies that a modest increase of cytoplasmic β-catenin can result in significant changes in signaling and cellular transformation and that overexpression of the wild-type gene alone is sufficient. This may also explain how the relatively small increase in endogenous cytoplasmic β-catenin that occurs before the onset of S phase may regulate the G_1/S transition in the normal cell cycle (Fig. 9 B). However, it is interesting to note that the increase in signaling above CON levels and the difference between the WT and S37A cells are relatively small when compared with other published results (Morin et al., 1997; Porfiri et al., 1997; Young et al., 1998). It is possible that the fact that this study was performed with cells that stably express a constitutively active transgene is responsible for both phenomena. We believe that the very high levels of β-catenin expression and signaling that can be achieved in nontransformed cells by transient transfection is not conducive to their survival and propagation. If true, selection pressures against very high expression would: result in the production of stable cells expressing only moderately elevated β-catenin protein and signaling; and, limit the extent to which the S37A mutation could stimulate signaling above WT β-catenin. In addition, some studies have used different β-catenin mutants, which may be more active.

It is plausible that some of the phenotypic alterations induced by β-catenin overexpression could be the result of altered cadherin function and independent of β-catenin signaling. However, the fact that these cells display strong intercellular adhesion at high density and retain the ability to generate tight junctions (as measured by electrical resistance across the monolayer in culture) demonstrates that E-cadherin function remains intact.

The APC/β-catenin signaling pathway has been implicated in a large number of epithelial cancers (Munemitsu et al., 1995; Inomata et al., 1996; Ilyas et al., 1997; Korinek

et al., 1997; Mareel et al., 1997; Morin et al., 1997; Peifer, 1997; Rubinfeld et al., 1997; Palacios and Gamallo, 1998; Voeller et al., 1998). In most cases, mutations in either APC or β-catenin result in stabilization of β-catenin protein and elevated β-catenin–LEF/TCF signaling. However, it is not clear what role this pathway has in normal cells. In this study, we demonstrate that β-catenin is a potent oncogene. All of the major phenomena that characterize cellular transformation, that is, soft agar growth, altered morphology, inhibition of apoptosis, and stimulation of cell cycle progression, can be induced by the modest overexpression of β-catenin in a nontransformed epithelial cell line. This clearly indicates that β-catenin can play a direct role in the process of carcinogenesis and that a major component of APC function is its downregulation. These data suggest that, as an early event in the progression of colorectal cancer, activation of β-catenin signaling promotes adenoma formation by promoting proliferation and survival of epithelial cells in the abnormal tissue architecture of a tumor mass. In addition, it may also promote the accumulation of mutations and cancer progression by attenuating the DNA damage-induced G_1 cell cycle block.

We would like to thank the following: Dr. Mark van de Wetering for supplying us with the TOPFLASH and FOPFLASH constructs; Manu Kohli for his help with the irradiation experiments; and Karen Creswell and Dr. Wen Hui Zhu of the Lombardi Cancer Research Center Flow Cytometry Core Facility for their help with all of the flow cytometry experiments.

This work was funded by grants from the United States Department of Defense.

Received: 8 December 1998
Revised: 23 June 1999
Accepted: 23 June 1999

References

Baeg, G., A. Matsumine, T. Kuroda, R.N. Bhattacharjee, I. Miyaashiro, K. Toyoshima, and T. Akiyama. 1995. The tumor suppressor gene product APC blocks cell cycle progression from G_0/G_1 to S phase. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:5618–5625.

Barth, A.I.M., A.L. Pollack, Y. Altschuler, K. Mostov, and W.J. Nelson. 1997. NH_2-terminal deletion of beta catenin results in stable colocalization of mutant beta catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J. Cell Biol.* 136:693–706.

Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M.M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive *v-SRC* gene. *J. Cell Biol.* 120:757–766.

Birchmeier, C., W. Birchmeier, and B. Brand-Saberi. 1996. Epithelial–mesenchymal transitions in cancer progression. *Acta Anat. (Basel).* 156:217–226.

Brancolini, C., D. Lazarevic, I. Rodriguez, and C. Schneider. 1997. Dismantling cell–cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. *J. Cell Biol.* 139:759–771.

Cadigan, K.M., and R. Nusse. 1997. Wnt signaling: a common theme in animal development. *Genes Dev.* 11:3286–3305.

Chen, C.S., M. Mrksich, S. Huang, G.M. Whitesides, and D.E. Ingber. 1997. Geometric control of cell life and death. *Science.* 276:1425–1428.

Clark, E.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science.* 268:233–239.

Clevers, H., and M. van de Wetering. 1997. TCF/LEF factor earn their wings. *Trends Genet.* 13:485–489.

Cook, D., M.J. Fry, K. Hughes, R. Sumathipala, J.R. Woodgett, and T.C. Dale. 1996. Wingless inactivates glycogen synthase kinase-3 via an intracellular signaling pathway which involves a protein kinase C. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:4526–4536.

Desbarats, L., A. Schneider, D. Muller, A. Burgin, and M. Eilers. 1996. Myc: a single gene controls both proliferation and apoptosis in mammalian cells. *Experientia.* 52:1123–1129.

Dietrich, C., K. Wallenfang, F. Oesch, and R. Wieser. 1997. Differences in the mechanisms of growth control in contact-inhibited and serum-deprived human fibroblasts. *Oncogene.* 15:2743–2747.

Evan, G.I., L. Brown, M. Whyte, and E. Harrington. 1995. Apoptosis and the cell cycle. *Curr. Opin. Cell Biol.* 7:825–834.

Fagotto, F., N. Funayama, U. Gluck, and B.M. Gumbiner. 1996. Binding to cadherin antagonizes the signaling activity of β-catenin during axis formation in *Xenopus. J. Cell Biol.* 132:1105–1114.

Frisch, S.M., and H. Francis. 1994. Disruption of epithelial cell–matrix interactions induces apoptosis. *J. Cell Biol.* 124:619–626.

Frisch, S.M., and E. Ruoslahti. 1997. Integrins and anoikis. *Curr. Opin. Cell Biol.* 9:701–706.

Frisch, S.M., K. Vuori, D. Kelaita, and S. Sicks. 1996a. A role for Jun-N-terminal kinase in anoikis; suppression by bcl-2 and crmA. *J. Cell Biol.* 135:1377–1382.

Frisch, S.M., K. Vuori, E. Ruoslahti, and P.Y. Chan-Hui. 1996b. Control of adhesion-dependent cell survival by focal adhesion kinase. *J. Cell Biol.* 134: 793–799.

Fukuchi, T., M. Sakamoto, H. Tsuda, K. Maruyama, S. Nozawa, and S. Hirohashi. 1998. Beta-catenin mutation in carcinoma of the uterine endometrium. *Cancer Res.* 58:3526–3528.

Gat, U., R. DasGupta, L. Degenstein, and E. Fuchs. 1998. *De Novo* hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell.* 95:605–614.

Giancotti, F.G. 1997. Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr. Opin. Cell Biol.* 9:691–700.

Gumbiner, B. 1997. Signal transduction by β-catenin. *Curr. Opin. Cell Biol.* 7:634–640.

He, T.C., A.B. Sparks, C. Rago, H. Hermeking, L. Zawel, L.T. da Costa, P.J. Morin, B. Vogelstein, and K.W. Kinzler. 1998. Identification of c-Myc as a target of the APC pathway. *Science.* 281:1509–1512.

Hirano, S., N. Kimoto, Y. Shimoyama, S. Hirohashi, and M. Takeichi. 1992. Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. *Cell.* 70:293–301.

Huber, O., C. Bierkamp, and R. Kemler. 1996. Cadherins and catenins in development. *Curr. Opin. Cell Biol.* 8:685–691.

Ilic, D., E.A.C. Almeida, D.D Schlaepfer, P. Dazin, S. Aizawa, and C.H. Damsky. 1998. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J. Cell Biol.* 143:547–560.

Ilyas, M., I.P. Tomlinson, A. Rowan, M. Pignatelli, and W.F. Bodmer. 1997. Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc. Natl. Acad. Sci. USA.* 94:10330–10334.

Inomata, M., A. Ochiai, S. Akimoto, S. Kitano, and S. Hirohashi. 1996. Alteration of beta catenin expression in colonic epithelial cells of familial adenomatous polyposis patients. *Cancer Res.* 56:2213–2217.

Kato, A., H. Takahashi, Y. Takahashi, and H. Matsushime. 1997. Contact inhibition-induced inactivation of the cyclin D-dependent kinase in rat fibroblast cell line, 3Y1. *Leukemia.* 11 Suppl 3:361–362.

Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* 9:317–321.

Korinek, V., N. Barker, P.J. Morin, D. van Wichen, R. de Weger, K.W. Kinzler, B. Vogelstein, and H. Clevers. 1997. Constitutive transcriptional activation by a β-catenin–Tcf complex in $APC^{-/-}$ colon carcinoma. *Science.* 275:1784–1787.

Mareel, M., T. Boterberg, V. Noe, L. Van Hoorde, S. Vermeulen, E. Bruyneel, and M. Bracke. 1997. E-cadherin/catenin/cytoskeleton complex: a regulator of cancer invasion. *J. Cell Physiol.* 173:271–274.

Mehta, P.P., J.S. Bertram, and W.R. Loewenstein. 1986. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell.* 44:187–196.

Miyoshi, Y., K. Iwao, Y. Nagasawa, T. Aihara, Y. Sasaki, S. Imaoka, M. Murata, T. Shimano, and Y. Nakamura. 1998. Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res.* 58:2524–2527.

Morin, P.J., B. Vogelstein, and K. Kinzler. 1996. Apoptosis and APC in colorectal tumorigenesis. *Proc. Natl. Acad. Sci. USA.* 93:7950–7954.

Morin, P.J., A.B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, and K.W. Kinzler. 1997. Activation of β-catenin/Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science.* 275:1787–1790.

Munemitsu, S., I. Albert, B. Souza, B. Rubinfeld, and P. Polakis. 1995. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc. Natl. Acad. Sci. USA.* 92:3046–3050.

Novak, A., S.C. Hsu, C. Leung-Hagesteijn, G. Radeva, J. Papkoff, R. Montesano, C. Roskelley, R. Grosschedl, and S. Dedhar. 1998. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc. Natl. Acad. Sci. USA.* 95:4374–4379.

Orford, K., C. Crockett, J.P. Jensen, A.M. Weissman, and S.W. Byers. 1997. Serine phosphorylation-regulated ubiquitination and degradation of beta catenin. *J. Biol. Chem.* 272:24735–24738.

Ozawa, M., H. Baribault, and R. Kemler. 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1711–1717.

Palacios, J., and C. Gamallo. 1998. Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res.* 58:1344–1347.

Papkoff, J., B. Rubinfeld, B. Schryver, and P. Polakis. 1996. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol. Cell. Biol.* 16: 2128–2134.

Peifer, M. 1995. Cell adhesion and signal transduction: the *Armadillo* connec-

tion. *Trends Cell Biol.* 5:224–229.
Peifer, M. 1997. Beta catenin as oncogene: the smoking gun. *Science.* 275:1752–1753.
Peifer, M., P.D. McCrea, K.J. Green, E. Wieschaus, and B. Gumbiner. 1992. The vertebrate adhesion junction proteins, beta catenin and plakoglobin and the *Drosophila* segment polarity gene *armadillo* form a multigene family with similar properties. *J. Cell Biol.* 118:681–691.
Peifer, M., D. Sweeton, M. Casey, and E. Wieschaus. 1994. *wingless* signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of *Armadillo*. *Development.* 120:369–380.
Polyak, K., J.Y. Kato, M.J. Solomon, C.J. Sherr, J. Massague, J.M. Roberts, and A. Koff. 1994. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev.* 8:9–22.
Porfiri, E., B. Rubinfeld, I. Albert, K. Hovanes, M. Waterman, and P. Polakis. 1997. Induction of a beta-catenin–LEF-1 complex by wnt-1 and transforming mutants of beta-catenin. *Oncogene.* 15:2833–2839.
Rubinfeld, B., B. Souza, I. Albert, O. Muller, S.H. Chamberlain, F.R. Masiarz, S. Munemitsu, and P. Polakis. 1993. Association of the APC gene product with beta-catenin. *Science.* 262:1731–1734.
Rubinfeld, B., P. Robbins, M. El-Gamil, I. Albert, E. Porfiri, and P. Polakis. 1997. Stabilization of β-catenin by genetic defects in melanoma cell lines. *Science.* 275:1790–1792.
Siegfried, E., T. Chou, and N. Perrimon. 1992. *wingless* signaling acts through *zeste-white 3*, the *Drosophila* homolog og *glycogen synthase kinase-3*, to regulate *engrailed* and establish cell fate. *Cell.* 71:1167–1179.
Simcha, I., M. Shtutman, D. Salomon, J. Zhurinsky, E. Sadot, B. Geiger, and Z. Ben. 1998. Differential nuclear translocation and transactivation potential of beta-catenin and plakoglobin. *J. Cell Biol.* 141:1433–1448.
Sommers, C.L., E.W. Thompson, J.A. Torri, R. Kemler, E.P. Gelmann, and S.W. Byers. 1991. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. *Cell Growth Differ.* 2:365–372.
Sommers, C.L., E.P. Gelmann, R. Kemler, P. Cowin, and S.W. Byers. 1994. Alterations in beta-catenin phosphorylation and plakoglobin expression in human breast cancer cell lines. *Cancer Res.* 54:3544–3552.
Stampfer, M.R., and J.C. Bartley. 1988. Human mammary epithelial cells in culture: differentiation and transformation. *Cancer Treat. Res.* 40:1–24.
Steiner, P., B. Rudolph, D. Muller, and M. Eilers. 1996. The functions of Myc in cell cycle progression and apoptosis. *Prog. Cell Cycle Res.* 2:73–82.
Su, L.-K., B. Vogelstein, and K.W. Kinzler. 1993. Association of the APC tumor suppressor protein with catenins. *Science.* 262:1734–1737.
Thompson, E.B. 1998. The many roles of c-Myc in apoptosis. *Annu. Rev. Physiol.* 60:575–600.
van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Louriero, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, et al. 1997. *Armadillo* coactivates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. *Cell.* 88:789–799.
Vindelov, L.L., I.J. Christensen, and N.I. Nissen. 1983. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry.* 3:323–327.
Voeller, H.J., C.I. Truica, and E.P. Gelmann. 1998. Beta-catenin mutations in human prostate cancer. *Cancer Res.* 58:2520–2523.
Weinert, T. 1998. DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. *Cell.* 94:555–558.
Wen, L.P., J.A. Fahrni, S. Troie, J.L. Guan, K. Orth, and G.D. Rosen. 1997. Cleavage of focal adhesion kinase by caspases during apoptosis. *J. Biol. Chem.* 272:26056–26061.
Whitehead, I., H. Kirk, and K. Kay. 1995. Expression cloning of oncogenes by retroviral transfer of cDNA libraries. *Mol. Cell. Biol.* 15:704–710.
Wong, M.H., B. Rubinfeld, and J.I. Gorden. 1998. Effects of forced expression of an NH_2-terminal truncated beta-catenin on mouse intestinal epithelial homeostasis. *J. Cell Biol.* 141:765–777.
Wu, C., S.Y. Keightley, C. Leung-Hagesteijn, G. Radeva, M. Coppolino, S. Goicoechea, J.A. McDonald, and S. Dedhar. 1998. Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J. Biol. Chem.* 273:528–536.
Young, C.S., M. Kitamura, S. Hardy, and J. Kitajewski. 1998. Wnt-1 induces growth, cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. *Mol. Cell. Biol.* 18:2474–2485.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 45, Issue of November 9, pp. 42276–42286, 2001
Printed in U.S.A.

Regulation of β-Catenin Function by the IκB Kinases*

Received for publication, May 10, 2001, and in revised form, August 28, 2001
Published, JBC Papers in Press, August 29, 2001, DOI 10.1074/jbc.M104227200

Carmela Lamberti‡§, Keng-Mean Lin‡§, Yumi Yamamoto‡§, Udit Verma‡, Inder M. Verma¶, Steven Byers‖, and Richard B. Gaynor‡**

From the ‡Division of Hematology-Oncology, Department of Medicine, Harold Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas 75390, ¶Salk Institute, La Jolla, California 92037, and the ‖Departments of Cell Biology and Oncology, Lombardi Cancer Center, Georgetown University School of Medicine, Washington, D. C. 20007

Both the β-catenin and the nuclear factor κB (NF-κB) proteins are important regulators of gene expression and cellular proliferation. Two kinases, IKKα and IKKβ, are critical activators of the NF-κB pathway. Here we present evidence that these kinases are also important in the regulation of β-catenin function. IKKα- and IKKβ-deficient mouse embryo fibroblasts exhibited different patterns of β-catenin cellular localization. IKKβ decreases β-catenin-dependent transcriptional activation, while IKKα increases β-catenin-dependent transcriptional activity. IKKα and IKKβ interact with and phosphorylate β-catenin using both *in vitro* and *in vivo* assays. Our results suggest that differential interactions of β-catenin with IKKα and IKKβ may in part be responsible for regulating β-catenin protein levels and cellular localization and integrating signaling events between the NF-κB and Wingless pathways.

β-Catenin, the mammalian homologue of the *Drosophila armadillo* protein, is a ubiquitously expressed protein that has at least two distinct roles in the cell. First, it participates in cell-cell adhesion by mediating the association of E-cadherin with the cytoskeleton (1, 2). Second, it is a critical downstream component of the Wnt[1] or Wingless signal transduction pathway (3–5). The Wnt family of secretory glycoproteins plays an important role in embryonic development, in the induction of cell polarity, and in the determination of cell fate. Deregulation of Wnt signaling disrupts axis formation in embryos (5–8) and is associated with multiple human malignancies (9).

The current model of Wnt signaling indicates that the binding of the Wnt proteins to their receptor, frizzled, stabilizes β-catenin by inhibiting the activity of a serine/threonine kinase glycogen synthase kinase-3 or GSK-3β (9). GSK-3β is associated with β-catenin in a multiprotein complex that includes the adenomatous polyposis coli tumor suppressor protein (APC), axin or conductin, protein phosphatase 2A, and dishevelled. GSK-3β phosphorylation of residues in the amino terminus of β-catenin results in APC-mediated β-catenin degradation via the ubiquitin-proteosome pathway (10, 11). Increased levels of β-catenin are frequently found in colon cancer due to mutations in either the APC gene (12–14) or at residues in the amino terminus of β-catenin that are phosphorylated by GSK-3β (15–17). In the nucleus, β-catenin forms a complex with members of the T-cell factor (TCF)/lymphocyte-enhancer factor (LEF) family and activates gene expression of a variety of target genes (18–23) including c-*myc* (24) and cyclin D1 (25, 26).

NF-κB comprises a family of transcription factors which are critical in activating the expression of genes involved in the immune and inflammatory response and in the regulation of cellular apoptosis (27, 28). NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins known as IκB. Upon stimulation of this pathway by a variety of agents including IL-1 and TNFα, the kinases IKKα and IKKβ (29–33) in conjunction with the scaffold protein IKKγ/NEMO (34–36) leads to the phosphorylation of IκBα at serine residues 32 and 36. Gene disruption studies in mice indicate that IKKβ appears to be the critical kinase in activating the NF-κB pathway (37–39), while IKKα appears to be critical for other functions such as keratinocyte differentiation (40–42). IKKα and IKKβ can form homodimers and also heterodimerize with each other, and this process is critical for their kinase activity. IKK phosphorylation of IκBα leads to its ubiquitination and degradation by the 26S proteasome and the nuclear translocation of NF-κB (43).

Interestingly, the sequence DSG*XX*S, which is the target site in IκB for IKK phosphorylation, is also found in the amino terminus of β-catenin (11). Phosphorylation of this sequence in both β-catenin and IκB stimulates their interactions with the ubiquitin ligase β-TrCP leading to their degradation by the proteasome (10, 11, 44). It has also been demonstrated that the β-catenin/TCF complex increases β-TrCP levels by a post-transcriptional mechanism to result in opposite effects on β-catenin and NF-κB activity (45). In addition, disruption of either the murine *GSK-3β* and *IKKβ* genes result in a similar phenotype with embryonic lethality due to hepatic apoptosis from increased sensitivity to TNFα (46). These results suggest potential relationships between β-catenin and NF-κB signaling pathways.

Given the fact that both the NF-κB and β-catenin pathways are important in the control of cellular proliferation and are regulated by cellular kinases that lead to β-TrCP-mediated degradation (10, 11, 45), we explored potential similarities in their regulation. First, we addressed whether there were differences in the cellular localization of β-catenin in wild-type mouse embryo fibroblasts as compared with fibroblasts derived from IKKα- and IKKβ-deficient mice. Next, we analyzed inter-

* This work was supported by National Institutes of Health Grant CA 74128. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

** To whom correspondence should be addressed: Div. of Hematology-Oncology, Dept. of Medicine, Univ. of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8594. Tel.: 214-648-4996; Fax: 214-648-4152; E-mail: gaynor@utsw.swmed.edu.

[1] The abbreviations used are: Wnt, Wingless; APC, adenomatous polyposis coli tumor suppressor protein; TCF, T-cell factor; LEF, lymphocyte-enhancer factor; NFκB, nuclear factor κB; IKK, IκB kinase; MEF, mouse embryo fibroblast; FITC, fluorescein isothiocyanate; NIK, NF-κB inducing kinase; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; HA, hemagglutinin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

actions between both IKKα and IKKβ and β-catenin and determined whether these kinases regulated β-catenin-dependent transcriptional activity. The results of this analysis indicate that IKKα can positively regulate β-catenin-dependent transcriptional activity while IKKβ negatively regulates this activity.

MATERIALS AND METHODS

Cells and Reagents—SW480 cells were purchased from American Tissue Culture Collection (Manassas, VA) and maintained in L-15 medium supplemented with 10% fetal bovine serum (HyClone Laboratories), 2 mM L-glutamine, and antibiotics (penicillin-streptomycin). COS, mouse embryo fibroblasts (MEFs, a kind gift of Xiaodong Wong), IKKα and IKKβ knock-out cells (39, 42) were maintained in Dulbecco's modified Eagle's medium and supplemented with the same components as above.

Antibodies—Polyclonal antibodies to IKKα (sc-7182), IKKβ (sc-7607), and β-catenin (sc-1496) were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against β-catenin and TFIIB (Transduction Laboratory), IKKα (PharMingen), the hemagglutinin epitope HA/12CA5 (Roche Molecular Biochemicals), and the FLAG-epitope-M2 (Sigma) were also used in immunoprecipitation and Western blot analysis. Donkey anti-rabbit, anti-mouse and anti-goat antibodies conjugated with either anti-FITC or Red-X rhodamine were obtained from Jackson Laboratory.

Plasmid Constructs—The pCMV5 expression plasmids containing either FLAG-tagged IKKα and IKKβ including the constitutively active kinases (SS/EE) with substitutions at residues 176/180 for IKKα or 177/181 for IKKβ and the kinase defective (K/M) mutants at residue 44 in both IKKα and IKKβ were described previously (30, 47, 48). Wild-type and mutant IKKα and IKKβ cDNAs were each cloned into the baculovirus expression vector pAcHLT. The recombinant baculoviruses were used to infect SF9 cells to produce recombinant IKK proteins for *in vitro* kinase assays (48). The pCMV5 expression vectors containing the wild-type and the dominant negative NIK mutant in which lysine residues at positions 429 and 430 were substituted with alanine contained an amino-terminal Myc-tag (48).

The pCMV5 expression vectors encoding full-length human β-catenin was provided by S. Byers, while the plasmids for LEF-1, TOPFLASH, and FOPFLASH were gifts of K. Kinzler and R. Grosschedl. The RSV-β-galactosidase construct was a gift from P. Chaudhary. The glutathione *S*-transferase (GST) full-length β-catenin (GST β-cat-(1–781)) bacterial expression vector was constructed by using polymerase chain reaction (PCR) to generate a fragment encompassing the full-length β-catenin, which was then cloned in frame with GST in the pGEX. The GST fusion protein containing the amino-terminal 91 amino acids of β-catenin was constructed by *Sac*I digestion and ligation of the GST fusion containing wild-type β-catenin. The constructs GST-β-cat-(130–781), GST-β-cat-(1–400), GST-β-cat-(130–400), and GST-β-cat-(618–781) were constructed using PCR. The amino-terminal deleted form of β-catenin utilized PCR primers to generate a fragment containing amino acids 130–781, which was cloned into pCMV5 and contained a carboxyl-terminal HA-epitope. All constructs that were generated by PCR were subjected to DNA sequencing and cloned into pcDNA3.

Expression and Purification of GST β-Catenin Fusion Proteins—Recombinant GST β-catenin fusion proteins were expressed in bacterial strain BL21 and lysed in HMK buffer (50 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride), and the bacterial lysates were incubated with 0.5 ml of packed glutathione-conjugated-agarose beads (Sigma) for 2 h at 4 °C. After three washes, the GST fusion proteins were eluted with 10 mM glutathione in HMK buffer and dialyzed, and protein purity was assessed by SDS-polyacrylamide gel electrophoresis.

Luciferase Reporter Assays—COS cells and mouse embryo fibroblasts were plated at 50% confluence in 35-mm tissue culture wells. After 24 h, the cells were transfected using LipofectAMINE Plus with the indicated DNA constructs and either the TOPFLASH luciferase reporter containing LEF/TCF binding sites or the FOPFLASH luciferase reporter with mutated LEF/TCF sites. An NF-κB luciferase reporter containing three NF-κB binding sites upstream of a thymidine kinase minimal promoter was used to detect NF-κB-directed gene expression. An RSV-β-galactosidase expression vector was included in the transfection assays to control for differences in transfection efficiency, and the pCMV5 plasmid was added to the transfection assays to standardize DNA quantities. Between 18 to 24 h posttransfection, the cells were washed twice with cold PBS, and the reporter activity was measured using the luciferase assay system (Promega). All transfections were done in duplicate and repeated at least three times.

Fractionation of Cellular Extracts—Cytoplasmic extracts were prepared from 10^8 SW480 or COS cells as described previously (49) with slight modifications. Cells were washed twice with cold PBS, and cell pellets were resuspended in 5 volumes of buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate, 0.5 μM okadaic acid, 1 mM sodium orthovanadate), and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, cells were lysed with 15 strokes of a Wheaton all-glass Dounce homogenizer (Tight pestle). Nuclei were pelleted by centrifugation for 5 min at 2000 rpm (Beckman bench-top centrifuge, CH3.7 rotor). The supernatants termed S100 were collected, mixed with 0.11 volume of buffer B (0.3 M Hepes (pH 7.9), 30 mM $MgCl_2$, and 1.4 M NaCl), and then centrifuged at 100,000 × *g* for 60 min at 4 °C.

Whole cell extracts were prepared from COS cells transfected with hemagglutinin-tagged β-catenin alone or β-catenin and FLAG-tagged IKKα and IKKβ as described (47) in lysis buffer containing 40 mM Tris, (pH 8), 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 5 mM β-glycerophosphate, 5 mM NaF, 1 mM $NaVO_4$ (pH 10.0), and protease inhibitors (Roche Molecular Biochemicals).

Gel Filtration Chromatography—S100 extracts prepared from the SW480 and COS cells were further fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) and equilibrated with buffer D (20 mM Hepes (pH 7.9), 0.1 M KCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20% glycerol). Protein markers (Sigma) used for the calibration of the column included bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.5 kDa).

Protein Association and Western Blot Analysis—For endogenous protein association studies, equal volumes of proteins (200–300 μl) from each of the Superdex-200 fraction were incubated overnight at 4 °C with 1 μg of indicated antibodies or normal mouse IgG followed by the addition of protein G-agarose (Sigma) for 2–3 h at 4 °C. For protein association studies using transfected IKK and β-catenin expression vectors, COS cells were transfected with FLAG-tagged IKKα or IKKβ and HA-tagged β-catenin cDNAs. Cells were harvested 18–24 h after transfection, extracts were prepared, and gel chromatography was performed as described above. Equal volumes of each column fraction were immunoprecipitated with 12CA5 antibody or anti-FLAG M2 antibody. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech), and probed with specific antibodies. The membrane-bound immune complexes were analyzed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech). For *in vitro* association studies, 40 μl of the cytoplasmic fractions were incubated overnight at 4 °C with 40 μl of the glutathione-conjugated-agarose bound with indicated proteins. Following three washes with 10 volumes of cold PBS, the protein complexes were subjected to Western blot analysis as described above.

In Vitro Kinase Assays—Kinase assays were performed as described by Yamamoto *et al.* (48). The baculovirus-produced IKK proteins were purified by nickel-agarose chromatography and then immunoprecipitated with 12CA5 monoclonal antibody (48). The epitope-tagged IKKα and IKKβ kinases were transfected into COS cells, and extracts were immunoprecipitated with the M2 monoclonal antibody directed against the FLAG-epitope. These kinases were added to kinase buffer containing 10 μCi of [γ-^{32}P]ATP, 1 mM ATP, 1 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), and then 1 μg of each of the substrates including wild-type or the S32A/S36A of GST-IκBα-(1–54) or GST-β-cat-(1–91) were incubated for 15 min at 30 °C. For determination of phosphate incorporation into the GST-IκBα-(1–54) and GST-β-catenin-(1–91), 2 μg of each of these substrates was incubated with either FLAG-tagged IKKα or IKKβ, which were immunoprecipitated from COS cell extract with the M2 monoclonal antibody in kinase buffer containing 15 μCi of [γ-^{32}P]ATP with a specific activity of 6000 Ci/mM (New England Nuclear) and either 0.01 mM, 0.01 mM, or 1.0 mM of cold ATP. The kinase reaction mixtures were subjected to electrophoresis on 10% SDS-polyacrylamide gels and autoradiography. The ^{32}P-labeled IκBα and β-catenin substrates were then subjected to scintillation counting, and the moles of phosphate incorporated were calculated. Reactions were incubated at 30 °C for 5, 15, 30, 60, and 120 min and stopped by the addition of protein loading buffer and heating to 90 °C.

Immunocytochemistry and Confocal Microscopy—Cells were cultured overnight on coverslips in Dulbecco's modified Eagle's medium without serum, washed two times with PBS, and fixed with 3.7% formaldehyde for 10 min followed by a brief permeabilization with 0.5% Triton X-100 in PBS. The cells were blocked for 30 min with 3% normal

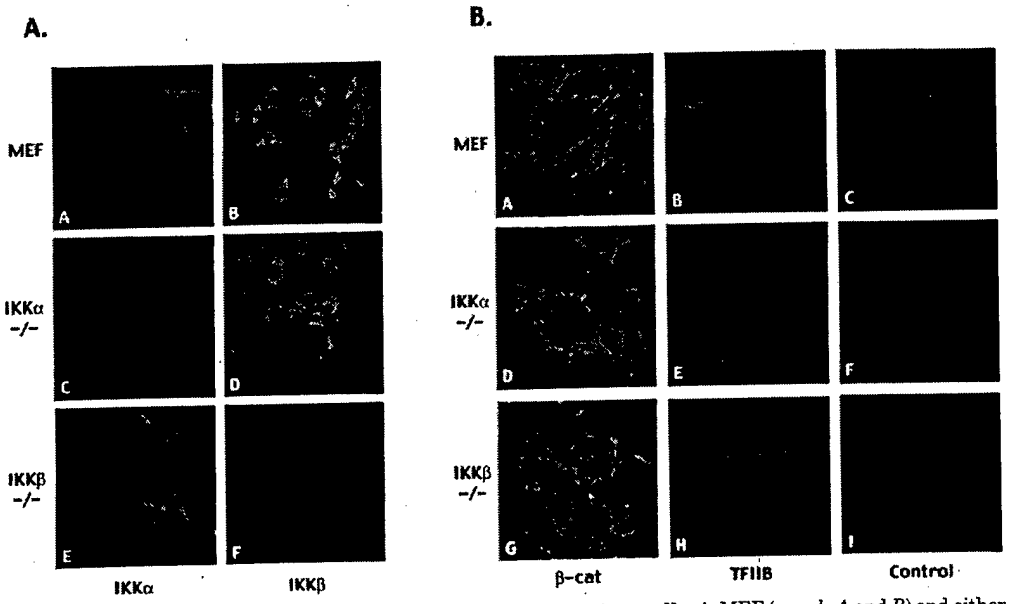


FIG. 1. **Characterization of β-catenin localization in IKKα- and IKKβ-deficient cells.** *A*, MEF (*panels A* and *B*) and either IKKα-deficient ($IKK\alpha^{-/-}$) (*panels C* and *D*) or IKKβ-deficient ($IKK\beta^{-/-}$) (*panels E* and *F*) embryo fibroblasts were plated overnight on coverslips before staining with either rabbit polyclonal antibodies directed against IKKα (*panels A, C,* and *E*) or IKKβ (*panels B, D,* and *F*) followed by staining with a secondary Red-X rhodamine-conjugated rabbit antibody. *B*, alternatively MEF (*panels A–C*), $IKK\alpha^{-/-}$ (*panels D–F*) and $IKK\beta^{-/-}$ (*panels G–I*) cells were stained with either a goat antibody to β-catenin (*green*) (*panels A, D,* and *G*) or a mouse monoclonal antibody to TFIIB (*red*) (*panels B, E,* and *H*). Donkey anti-goat antibody conjugated with FITC and anti-mouse conjugated with Red-X rhodamine were used as secondary antibodies. The respective negative controls utilizing the TFIIB monoclonal antibody and the donkey anti-goat FITC-conjugated antibody are also shown (*panels C, F,* and *I*). Images were collected using a laser scanning confocal microscope (Bio-Rad).

donkey serum in PBS and then incubated for 1 h with primary antibodies (diluted 1:50 to 1:200 in 1% normal donkey serum in PBS). The coverslips were washed three times with PBS and then incubated for 1 h with the secondary antibodies conjugated with FITC or Red-X rhodamine (diluted 1:400 in 1% normal donkey serum in PBS). Samples were washed three times and then treated with Aquamount (Polysciences). The results were analyzed on a laser scanning confocal microscope MRC 1000 (Bio-Rad).

RESULTS

β-Catenin Localization in IKK-deficient Cells—First, the localization of IKKα and IKKβ in wild-type MEFs was compared with that seen in IKKα-deficient ($IKK\alpha^{-/-}$) and IKKβ-deficient ($IKK\beta^{-/-}$) cells using immunofluorescence analysis with confocal microscopy. Wild-type mouse embryo fibroblasts (Fig. 1*A*, *panels A* and *B*), $IKK\alpha^{-/-}$ (Fig. 1*A*, *C* and *D*), and $IKK\beta^{-/-}$ (Fig. 1*A*, *panels E* and *F*) cells were plated on coverslips overnight and stained with rabbit polyclonal antibodies directed against either IKKα or IKKβ. In MEFs, IKKα localized in both the nucleus and the cytoplasm, while IKKβ localized predominantly in the cytoplasm (Fig. 1*A*, *panels A* and *B*). In $IKK\alpha^{-/-}$ cells, IKKβ localized predominantly in the cytoplasm (Fig. 1*A*, *panel D*). In $IKK\beta^{-/-}$ cells, there appeared to be increased IKKα present in the nucleus as compared with that seen in MEFs (Fig. 1*A*, *panel E*). There was no IKKα staining observed in $IKK\alpha^{-/-}$ cells (Fig. 1*A*, *panel C*) or IKKβ staining seen in $IKK\beta^{-/-}$ (Fig. 1*A*, *panel F*), thus confirming the identity of these cells.

Immunostaining of MEFs, IKKα-, and IKKβ-deficient cells with a polyclonal antibody directed against β-catenin demonstrated that β-catenin has a different pattern of staining in $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ cells. β-Catenin was present in both the nucleus and the cytoplasm of MEFs with marked accumulation at cell-cell junctions (Fig. 1*B*, *panel A*). In $IKK\alpha^{-/-}$ cells, there was reduced nuclear staining of β-catenin as compared with MEF cells (Fig. 1*B*, *panel G*). There was more β-catenin present in the nucleus and the perinuclear region of $IKK\beta^{-/-}$ cells than in $IKK\alpha^{-/-}$ cells (Fig. 1*B*, *panel G*). As a control, these cells were also stained with a monoclonal antibody directed against the basal transcription factor TFIIB, which is localized predominantly in the nucleus (Fig. 1*B*, *panels B, E,* and *H*). There was little background staining when the FITC-conjugated anti-goat secondary antibody was used with the mouse monoclonal antibody directed against TFIIB (Fig. 1*B*, *panels C, F,* and *I*). These results indicate that there is less β-catenin localized in the nucleus of $IKK\alpha^{-/-}$ cells than in either $IKK\beta^{-/-}$ cells or MEF cells.

β-Catenin Activity in IKK-deficient Cells—Next we addressed whether the differences in β-catenin distribution in the IKK-deficient embryo fibroblasts could alter its transcriptional activity. The IKK-deficient cells and the parental MEFs were transfected with a TOPFLASH reporter construct alone or with expression vectors encoding either LEF-1 or β-catenin. The TOPFLASH reporter is driven by four LEF/TCF binding motifs inserted upstream of a minimal c-*fos* promoter and a luciferase gene (15). As a control, the FOPFLASH reporter, which lacks LEF/TCF binding sites, was utilized. An RSV-β-galactosidase expression vector was included in these transfections to control for differences in transfection efficiency.

When the TOPFLASH reporter alone was transfected into $IKK\alpha^{-/-}$ cells, there was consistently a 5–6-fold lower level of activity as compared with that observed in $IKK\beta^{-/-}$ cells (Fig. 2). Transfection of an expression vector encoding LEF-1 into either $IKK\alpha^{-/-}$ or $IKK\beta^{-/-}$ cells markedly stimulated TOPFLASH activity as did transfection of expression vectors encoding both LEF-1 and β-catenin. There was no significant activity from the FOPFLASH reporter in either the absence or presence of β-catenin and LEF-1 (Fig. 2). Transfection of expression vectors encoding wild-type IKKβ and LEF-1 into $IKK\alpha^{-/-}$ cells reduced TOPFLASH activity, while transfection of an IKKα expression vector with LEF-1 increased TOPFLASH activity in these cells (Fig. 2). When similar studies were performed in $IKK\beta^{-/-}$ cells, transfection of an IKKβ expression vector reduced TOPFLASH activity, while transfection of an IKKα ex-

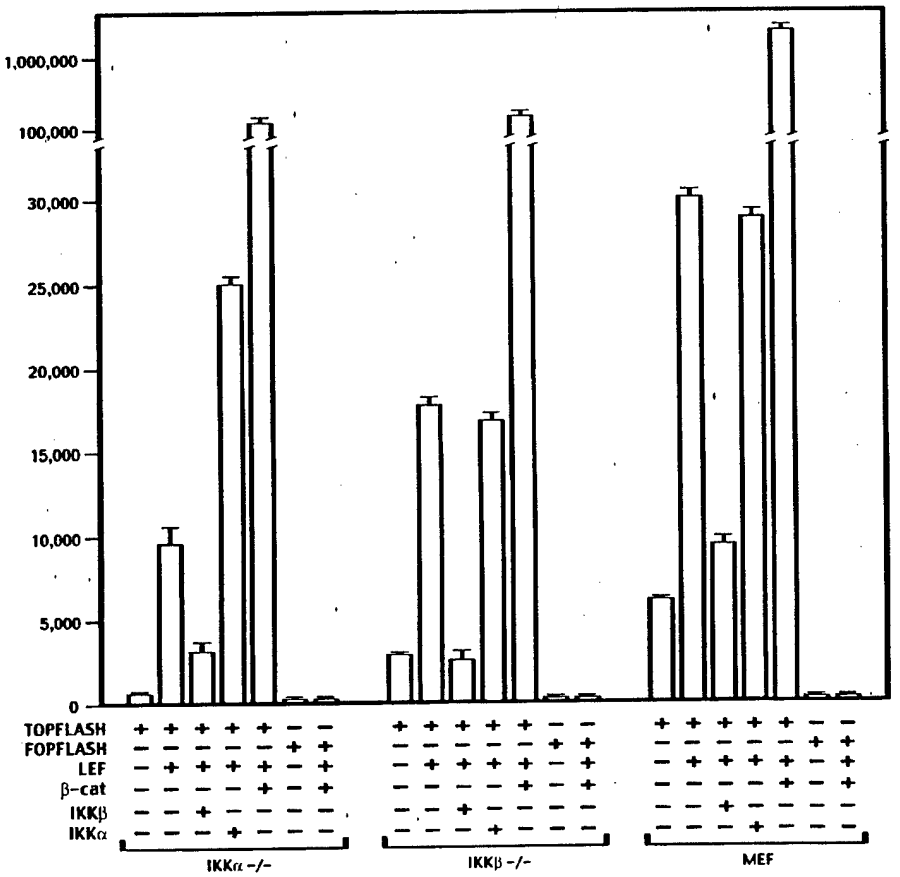


FIG. 2. **β-Catenin-mediated gene expression in IKKα- and IKKβ-deficient cells.** IKKα$^{-/-}$, IKKβ$^{-/-}$, and MEFs were each cotransfected with either a TOPFLASH or FOPFLASH reporter (0.85 μg) and pCMV5 expression vectors encoding β-catenin (0.5 μg), LEF-1 (50 ng), and either IKKα or IKKβ (0.5 μg) as indicated and an RSV-β-galactosidase reporter (0.60 μg) using LipofectAMINE Plus (Life Technologies, Inc.). All transfections contained 2.5 μg of DNA with a pCMV5 expression vector added to standardize DNA quantities. After 18 h, the cells were collected and lysed, both luciferase and β-galactosidase activity was determined, and the normalized luciferase activity was calculated by correcting for differences in β-galactosidase activity.

pression vector did not significantly alter TOPFLASH activity (Fig. 2). Transfection of both β-catenin and LEF-1 resulted in similar levels of TOPFLASH activity in the IKKβ$^{-/-}$ and IKKα$^{-/-}$ cells (Fig. 2). The parental MEF cells consistently gave somewhat higher TOPFLASH activity than seen in the IKK-deficient cells (Fig. 2). Again transfection of an IKKβ expression vector with LEF-1 into these cells reduced TOPFLASH activity, while transfection of an IKKα expression vector with LEF-1 resulted in little change in TOPFLASH activity (Fig. 2). Thus, the reduced levels of endogenous β-catenin in the nuclei of IKKα$^{-/-}$ cells are associated with decreased β-catenin activation of gene expression, and this defect could be complemented by transfection of an IKKα expression vector. IKKα does not increase gene expression in the IKKβ$^{-/-}$ and MEF cells, which have relatively abundant levels of nuclear β-catenin.

IKKβ and IKKα Have Differential Effects on β-Catenin Transactivation—The results presented in the previous section suggested that IKKβ and IKKα could potentially be involved in regulating the transcriptional stimulatory properties of β-catenin. Thus it was important to address whether either IKKα or IKKβ could alter β-catenin-mediated transcriptional activation in COS cells, which have low levels of endogenous β-catenin in the nucleus and relatively low levels of IKKα and IKKβ (data not shown). COS cells were transfected with either a TOPFLASH or FOPFLASH reporter, LEF-1 and β-catenin expression vectors, and increasing amounts of expression vectors encoding either the wild-type, constitutively active or kinase-defective mutants of IKKα and IKKβ. The constitutively active IKK proteins (IKKα SS/EE and IKKβ SS/EE) have glutamate substituted for serine residues in their T-loop so as to mimic phosphorylation of these residues and increase the activity of these kinases (30). The kinase-defective mutants (IKKα K/M and IKKβ K/M) contain a substitution of a lysine residue at position 44 with methionine (30).

As previously demonstrated, the coexpression of β-catenin and LEF-1 increased TOPFLASH but not FOPFLASH activity (Fig. 3*A*). When either wild-type IKKβ or the constitutively activate kinase, IKKβ SS/EE, was cotransfected with β-catenin and LEF-1, TOPFLASH activity decreased in a concentration-dependent manner (Fig. 3*A*). In contrast, cotransfection of either wild-type IKKα or the constitutively active kinase, IKKα SS/EE, increased β-catenin-dependent transactivation in a concentration-dependent manner (Fig. 3*A*). Transfection of the IKKβ K/M mutant resulted in a modest decrease in β-catenin transactivation that was not concentration-dependent, while transfection of the IKKα K/M mutant did not significantly alter β-catenin transactivation (Fig. 3*A*).

The cotransfection experiments in COS cells indicated that IKKα increased β-catenin-dependent gene expression, while IKKβ decreased β-catenin-dependent gene expression. Thus, we investigated whether IKKα and IKKβ can alter β-catenin protein levels. In addition, we asked whether IKKα and IKKβ

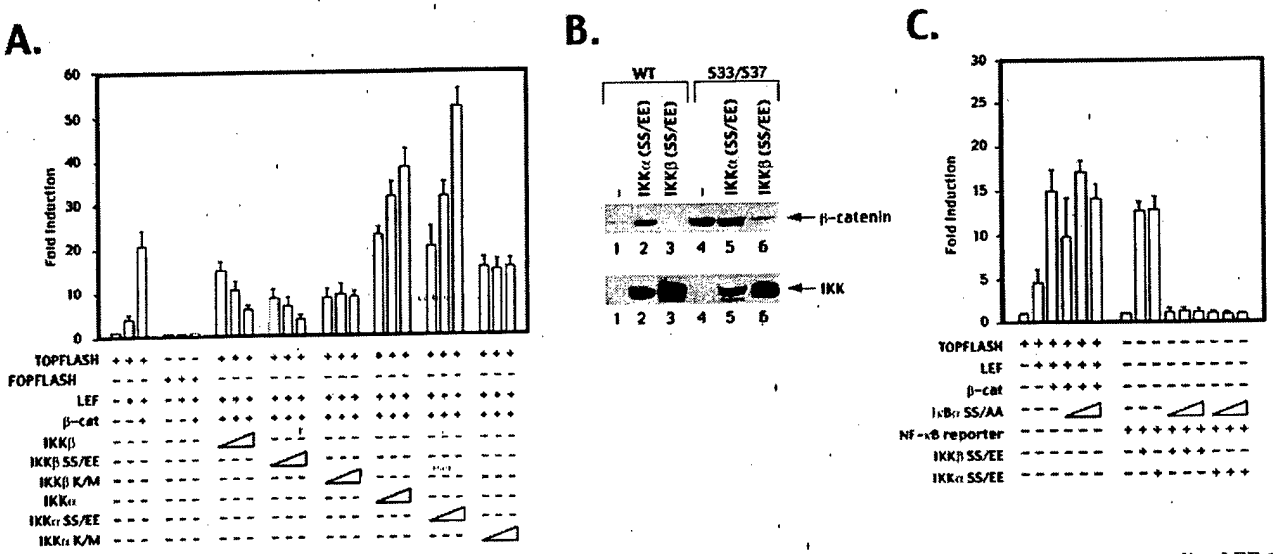


FIG. 3. **Role of IKKα and IKKβ in β-catenin transactivation.** *A*, the TOPFLASH reporter (0.75 μg) and expression vectors encoding LEF-1 (50 ng) and β-catenin (0.7 μg) were cotransfected into COS cells in the presence of wild-type IKKα or IKKβ, the constitutively active kinases, IKKβ SS/EE and IKKα SS/EE, or the kinase-defective mutants, IKKβ K/M and IKKα K/M, utilizing either 0.2, 0.5 or 1.0 μg of these kinases. *B*, COS cells were transfected with pCMV5 expression vectors encoding either HA-tagged wild-type (*lanes 1–3*) or the S33A/S37A mutant of β-catenin (0.7 μg) and pCMV5 (1.0 μg) (*lanes 1* and *3*) or either wild-type or mutant β-catenin together with an expression vector encoding the constitutively active FLAG-tagged IKKα (1.0 μg) (*lanes 2* and *4*) or FLAG-tagged IKKβ (1.0 μg) (*lanes 3* and *6*). Whole cell extracts were prepared and subjected to Western blot analysis with the anti-HA monoclonal antibody 12CA5 to detect the HA-tagged β-catenin (*lanes 1–6, top panel*) or the M2 monoclonal antibody directed against the FLAG-epitope to detect FLAG-tagged IKKα and IKKβ (*lanes 1–6, lower panel*). *C*, the TOPFLASH reporter was cotransfected with expression vectors encoding LEF-1 and β-catenin and the IκBα super-repressor (0.1, 0.2 and 0.5 μg) vectors. In addition, the NF-κB luciferase reporter (100 ng) was transfected with expression vectors encoding either the constitutively active IKKβ SS/EE or IKKα SS/EE and the IκBα super-repressor. All COS cell transfections contained 0.5 μg of an RSV-β-galactosidase expression vector, and the DNA amounts were standardized with a pCMV5 vector. Extracts were prepared 18 h after transfection, and the normalized luciferase activity was determined by correcting for differences in β-galactosidase activity. The change in gene expression relative to the TOPFLASH reporter alone was determined for each transfection and the average of three experiments (each in duplicate) is shown.

would affect the protein levels of a β-catenin mutant in which serine residues 33 and 37 were changed to alanine to result in increased β-catenin protein levels (15–17). Expression vectors encoding either the hemagglutinin-tagged wild-type or S33A/S37A mutant β-catenin were transfected into COS cells either alone or in the presence of either the constitutively active FLAG-tagged IKKα or IKKβ. Whole cell extracts were prepared from the transfected COS cells and analyzed by Western blot analysis using the 12CA5 and M2 monoclonal antibodies directed against the hemagglutinin and FLAG epitopes, respectively (Fig. 3*B*). IKKα expression increased the level of the epitope-tagged β-catenin protein (Fig. 3*B*, *lane 2*), while IKKβ reduced the amount of the epitope-tagged β-catenin protein (Fig. 3*B*, *lane 3*). In contrast, IKKα did not alter the level of the S33A/S37A β-catenin mutant (Fig. 3*B*, *lane 5*), while IKKβ reduced the level of this protein (Fig. 3*B*, *lane 6*). Transfection assays with the TOPFLASH reporter indicated that IKKα increased gene expression in the presence of the wild-type but not the mutant β-catenin, while IKKβ reduced gene expression in the presence of both of these β-catenin proteins (data not shown). These results suggest that IKKα either directly or indirectly may lead to increased levels of β-catenin to increase TOPFLASH activity, while IKKβ may reduce the levels of β-catenin to decrease β-catenin activity. The failure of IKKα to further increase the protein levels of the mutant β-catenin suggests that the structure of the amino terminus of β-catenin may be important in this process.

It was important to determine whether activation of the NF-κB pathway may be involved in the increased TOPFLASH activity seen in the presence of β-catenin and LEF-1. The TOPFLASH reporter was transfected with expression vectors encoding β-catenin, LEF-1, and the IκBα super-repressor (IκBα SS/AA) (Fig. 3*C*). The IκBα super-repressor protein, which contains substitutions of serine residues 32 and 36 with alanine, cannot be phosphorylated by IKK, and its resistance to degradation prevents the nuclear translocation of the NF-κB proteins in response to activators of this pathway (27). The transfection of the IκBα super-repressor did not alter activation of the TOPFLASH reporter in the presence of β-catenin and LEF-1 expression vectors, while it completely abolished the activity of an NF-κB reporter (Fig. 3*C*). These results suggest that NF-κB activation does not appear to be involved in the activation of TOPFLASH activity by β-catenin and LEF-1.

The Amino Terminus of β-Catenin Is Critical for IKKα but Not IKKβ Modulation of Gene Expression—Next we addressed whether the same or different domains in β-catenin were required for regulation by IKKα and IKKβ. The amino terminus of β-catenin is phosphorylated by GSK-3β leading to β-catenin degradation (50–52). Amino-terminal deletion mutants of β-catenin are very stable because they lack sequences that are involved in APC-mediated degradation (50–54). Furthermore, our results suggested that the amino terminus of β-catenin may be involved in IKKα-mediated regulation. To determine whether the amino terminus of β-catenin was critical for mediating the effects of IKKα and IKKβ, transfection of increasing amounts of a β-catenin expression vector deleted of its first 129 amino acids was transfected into COS cells along with LEF-1. There was increased TOPFLASH activity seen with this mutant similar to the results seen with wild-type β-catenin (Fig. 4). The expression of the constitutively active IKKβ protein reduced activation of TOPFLASH reporter when transfected with this β-catenin mutant. In contrast, the expression of the constitutively active IKKα protein did not alter the ability of the amino-terminal deletion of β-catenin to activate the TOPFLASH reporter (Fig. 4). These results suggest that the decreased β-catenin transactivation observed with IKKβ is not dependent on the amino terminus of β-catenin, while IKKα requires the presence of this domain to stimulate β-catenin transcriptional activity.

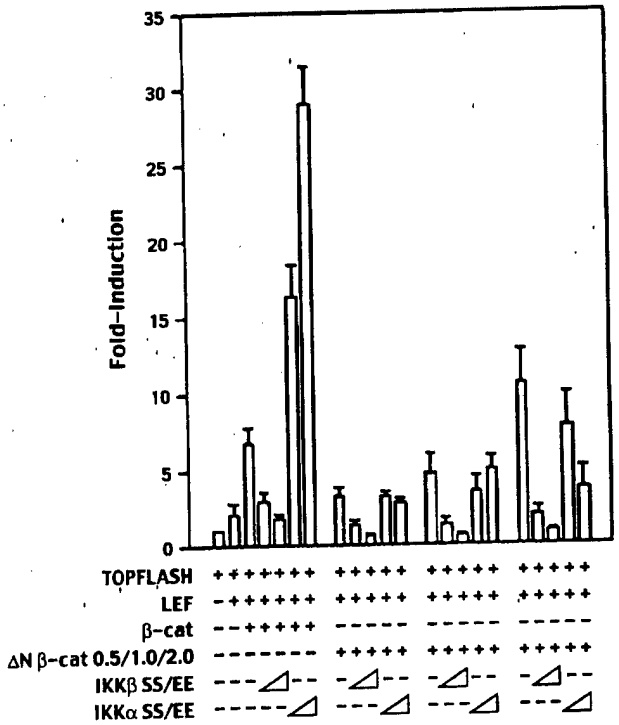


FIG. 4. **Differential effects of IKKβ and IKKα on transactivation of an amino-terminal truncated β-catenin.** COS cells were cotransfected with the indicated plasmids including the TOPFLASH reporter, LEF-1, and either wild-type β-catenin (*group 1*) or an amino-terminal deletion of the first 129 amino acids of β-catenin (*groups 2–4*). The construct encoding the amino-terminal-deleted β-catenin was cotransfected at concentrations of 0.5 (*group 2*), 1.0 (*group 3*), and 2.0 μg (*group 4*) together with the constitutively active kinases IKKβ SS/EE (0.5 and 1.0 μg) and IKKα SS/EE (0.5 and 1.0 μg). An RSV-β-galactosidase expression vector was added to each transfection, and DNA quantities were standardized by addition of a pCMV5 expression vector. After 18 h, the cells were collected and luciferase activity was determined and normalized to correct for differences in β-galactosidase activity. The change in gene expression relative to the TOPFLASH reporter alone was determined for each transfection and the average of three experiments (each in duplicate) is presented.

β-Catenin Interacts with IKKα and IKKβ—To address whether the effects of IKKα and IKKβ on β-catenin-dependent gene expression may be mediated by direct interactions with β-catenin, we performed coimmunoprecipitation experiments of β-catenin and the IKK proteins using cytoplasmic extracts prepared from the SW480 colon cancer cell line. SW480 cells express a truncated APC gene product and result in enhanced levels of β-catenin. This increased level of β-catenin was necessary to detect this protein in Western blot analysis of column fractions that were generated following chromatography (14).

Superdex-200 gel filtration chromatography of the S100 cytoplasmic extract prepared from SW480 cells was utilized to assay interactions between the IKK and β-catenin. Similar chromatographic analysis has previously been used to characterize the high molecular weight IKK complex (55). Western blot analysis of these column fractions indicated that β-catenin was present in a broad peak, including a portion that was present in high molecular weight fractions that also contained IKKα and IKKβ (Fig. 5*A*, *left panel*). Column fractions 7–12, which contained both β-catenin and the IKK proteins, were immunoprecipitated with a monoclonal antibody directed against β-catenin followed by Western blot analysis with either β-catenin, IKKα, or IKKβ antibodies (Fig. 5*A*, *middle panel*). This analysis indicated that β-catenin was associated with IKKα and IKKβ, while immunoprecipitation of these column fractions with mouse IgG followed by Western blot analysis demonstrated no association of these proteins (Fig. 5*A*, *right panel*). These results suggest that endogenous β-catenin can associate with IKKα and IKKβ.

Next, we characterized the interactions of IKKα and IKKβ with β-catenin following cotransfection of COS cells with expression vectors encoding these epitope-tagged proteins (Fig. 5*B*). First, S100 extracts prepared from these cells were subjected to Superdex-200 chromatography and Western blot analysis. As previously noted, when IKKα and IKKβ were transfected into COS cells they migrate in a relatively broad peak following Superdex-200 chromatography due to the failure to completely assemble into the high molecular weight IKK complex (49) (Fig. 5*B*, *left* and *middle panels*). Next, immunoprecipitation of column fractions prepared from extracts containing the FLAG-tagged IKKα and HA-tagged β-catenin was performed followed by Western blotting. This analysis indicated that FLAG-tagged IKKα and HA-tagged β-catenin were able to associate (Fig. 5*B*, *left panel*). Column fractions of extracts prepared from COS cells cotransfected with FLAG-tagged IKKβ and HA-tagged β-catenin indicated that both of these proteins were also able to associate (Fig. 5*B*, *middle panel*). Western blot analysis of the immunoprecipitated IKK and β-catenin proteins do not strictly overlap. This is likely due to the fact that their elution profiles following chromatography vary, which is consistent with the presence of these proteins in multiple complexes. The column fractions containing HA-tagged β-catenin and either FLAG-tagged IKKα or IKKβ were also immunoprecipitated with mouse IgG and analyzed by Western blot analysis. This analysis revealed that there were not nonspecific associations of the β-catenin and IKK proteins (Fig. 5*B*, *right panel*).

To further characterize the interactions of β-catenin with IKKα and IKKβ, *in vitro* binding of SW480 cytoplasmic extract with GST proteins fused to different domains of β-catenin was performed. Thus, we could determine the role of different domains of β-catenin including the amino terminus, which regulates protein stability, the armadillo repeats, and the C-terminal transactivation domain in binding the IKK proteins (53). Following the incubation of the SW480 cytoplasmic extract with the GST-β-catenin fusion proteins bound to glutathione-Sepharose beads, Western analysis was performed with antibodies directed against either IKKα or IKKβ. Each of the β-catenin fusion proteins, but not GST alone, was able to interact with IKKα and IKKβ (Fig. 6*B*). However, the GST-β-catenin fusion proteins extending between amino acid residues 1–400 and 130–400 consistently bound more IKKα and IKKβ (Fig. 6*B*). These results suggested that the region of β-catenin containing the first six armadillo repeats was probably critical for interaction with the IKK proteins. The data from the GST-pull down assays in conjunction with coimmunoprecipitation data of both endogenous and transfected proteins demonstrate that the IKK proteins and β-catenin can interact under a variety of different conditions.

IKKα and IKKβ Phosphorylate β-Catenin—Next we addressed whether IKK could phosphorylate the amino terminus of β-catenin and whether stimulation of IKK activity could result in increased β-catenin phosphorylation in *in vitro* kinase assays. The amino terminus of β-catenin has been demonstrated to be a target for GSK-3β phosphorylation (9), while serine residues 32 and 36 in the amino terminus of GST-IκBα are the target for IKK phosphorylation (29–33). HeLa cells were either untreated, treated with TNFα, or transfected with an expression vector encoding NIK (56, 57) to induce IKK kinase activity. The IKK complex was immunoprecipitated from extracts prepared from these cells and assayed for its

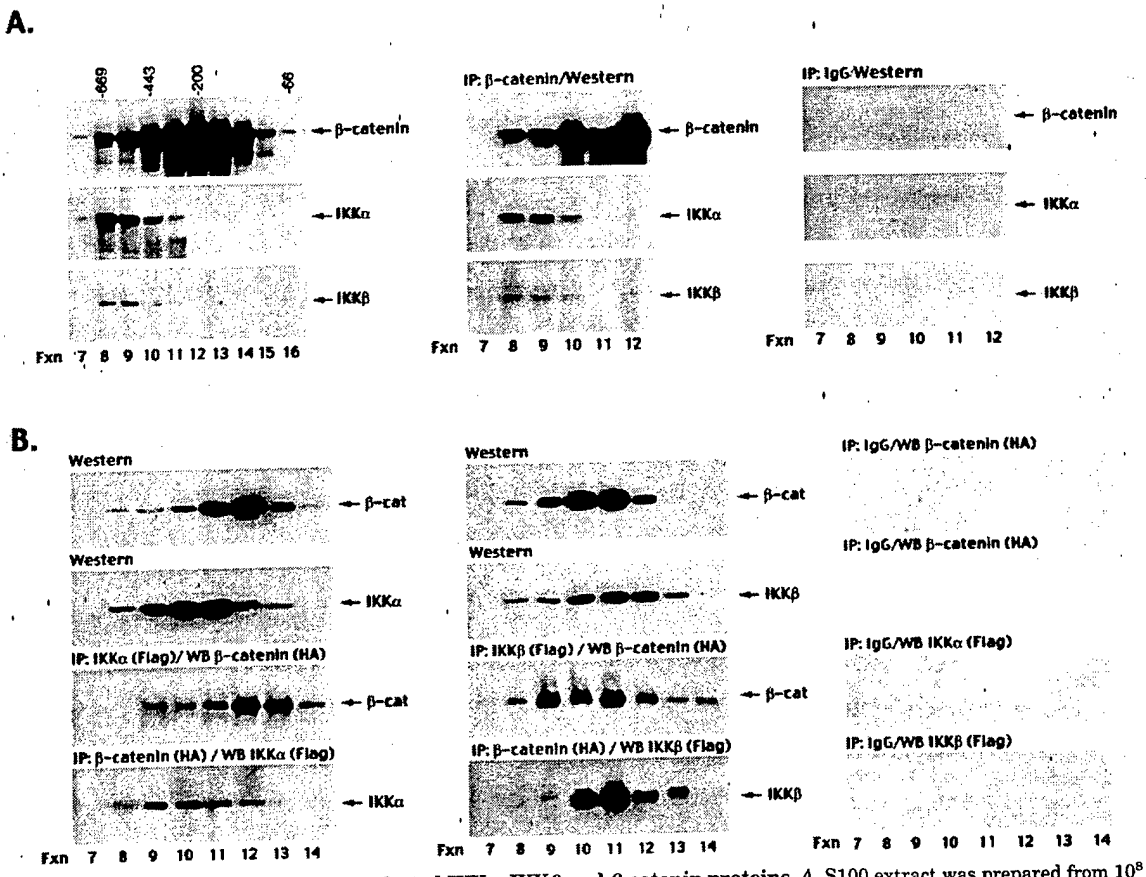


FIG. 5. **Association of endogenous and transfected IKKα, IKKβ and β-catenin proteins.** *A*, S100 extract was prepared from 10^8 SW480 cells and fractionated on a Superdex-200 gel filtration column. Equal volumes (40 μl) from each fraction were immunoblotted to monitor distribution of β-catenin, IKKα, and IKKβ (*left panel*). The mobility of the different protein markers on the Superdex-200 column are indicated as are the column fraction numbers. For association studies, equal volumes (200 μl) from *column fractions 7–12*, which contained the β-catenin and the IKK proteins, were incubated overnight with monoclonal antibodies directed against β-catenin (Transduction Laboratories). Western blot analysis was then performed on these immunoprecipitates using polyclonal goat antibody to detect β-catenin or rabbit polyclonal antibodies to detect IKKα and IKKβ (*middle panel*). These column fractions were also immunoprecipitated with mouse IgG followed by Western blot analysis with polyclonal antibodies directed against either β-catenin, IKKα, or IKKβ (*right panel*). *B*, COS cells were transfected using LipofectAMINE Plus with expression vectors encoding HA-tagged β-catenin and either FLAG-tagged IKKα or FLAG-tagged IKKβ. Cells were harvested 18 h after transfection, and S100 extracts were prepared and fractionated on a Superdex-200 column. Equal column volumes (40 μl) were subjected to Western blot analysis using 12CA5 antibody to detect HA-tagged β-catenin (*left* and *middle panels*) or the M2 monoclonal antibody to detect FLAG-tagged IKKα (*left panel*) or FLAG-tagged IKKβ (*middle panel*). Immunoprecipitation of column fractions prepared from FLAG-tagged IKKα and HA-tagged β-catenin (*left panel*) or FLAG-tagged IKKβ and HA-tagged β-catenin (*middle panel*) transfected cells was performed using either the 12CA5 or M2 monoclonal antibodies followed by Western blot analysis with the antibody that was not used in the immunoprecipitation. Column fractions from extracts containing either FLAG-tagged IKKα and HA-tagged β-catenin (*right panel*, *first* and *third gels*) or FLAG-tagged IKKβ and HA-tagged β-catenin (*right panel*, *second* and *fourth gels*) were immnoprecipitated with mouse IgG followed by Western blot analysis with either 12CA5 (*HA*) or M2 (*FLAG*) antibodies as indicated.

ability to phosphorylate either GST-β-cat-(1–91), GST-IκBα-(1–54), or GST-IκBα (SS/AA)-(1–54). IKK activity was induced by treatment with either TNFα or NIK and increased the phosphorylation of β-catenin (Fig. 7*A*, *lanes 1–3*) and IκBα (Fig. 7*A*, *lanes 4–6*), but not the IκBα mutant in which serine residues 32 and 36 were changed to alanine (Fig. 7*A*, *lanes 7–9*).

Recombinant baculovirus-produced IKKα and IKKβ were also tested in *in vitro* kinase assays using GST fusions with β-catenin or IκBα. Both IKKα and IKKβ also phosphorylated the amino terminus of β-catenin and IκBα, but not the IκBα mutant (Fig. 7*B*). COS cells were next transfected with either epitope-tagged wild-type or mutant IKKα and IKKβ, and following immunoprecipitation with the M2 monoclonal antibody these kinases were assayed using *in vitro* kinase assays with β-catenin and IκBα as substrates (48). Wild-type IKKα and IKKβ, but not the kinase-defective mutants, were able to phosphorylate β-catenin and IκBα (Fig. 7*C*).

Finally, we addressed whether IKKα and IKKβ could also phosphorylate additional regions in β-catenin other than its amino terminus (Fig. 7*D*). Both kinases phosphorylated GST fusion proteins containing various portions of β-catenin (Fig. 7*D*, *lanes 2–5*). These GST fusions contained either the amino terminus of β-catenin, an amino-terminal-deleted form of β-catenin or full-length β-catenin (Fig. 7*D*). Similar results were obtained using IKKα and IKKβ preparations produced by baculovirus expression (data not shown). These results indicate that both IKKα and IKKβ phosphorylate multiple regions of β-catenin.

Stoichiometry of IKK Phosphorylation of IκBα and β-Catenin—Next we compared the ability of IKKα and IKKβ to phosphorylate GST-IκBα-(1–54) and GST-β-cat-(1–91) substrates. In these *in vitro* kinase assays, we analyzed the phosphorylation of each of these substrates at specific points over a 120-min time course utilizing 0.01 mM, 0.1 mM, and 1.0 mM of cold ATP and 15 μCi of [γ-^{32}]ATP. Following SDS-PAGE and autoradiography (Figs. 8*A* and 8*B*, *top panels*), the ^{32}P-incorporation into the β-catenin and IκBα substrates was determined, and the number of the moles of phosphate incorporated per mole of

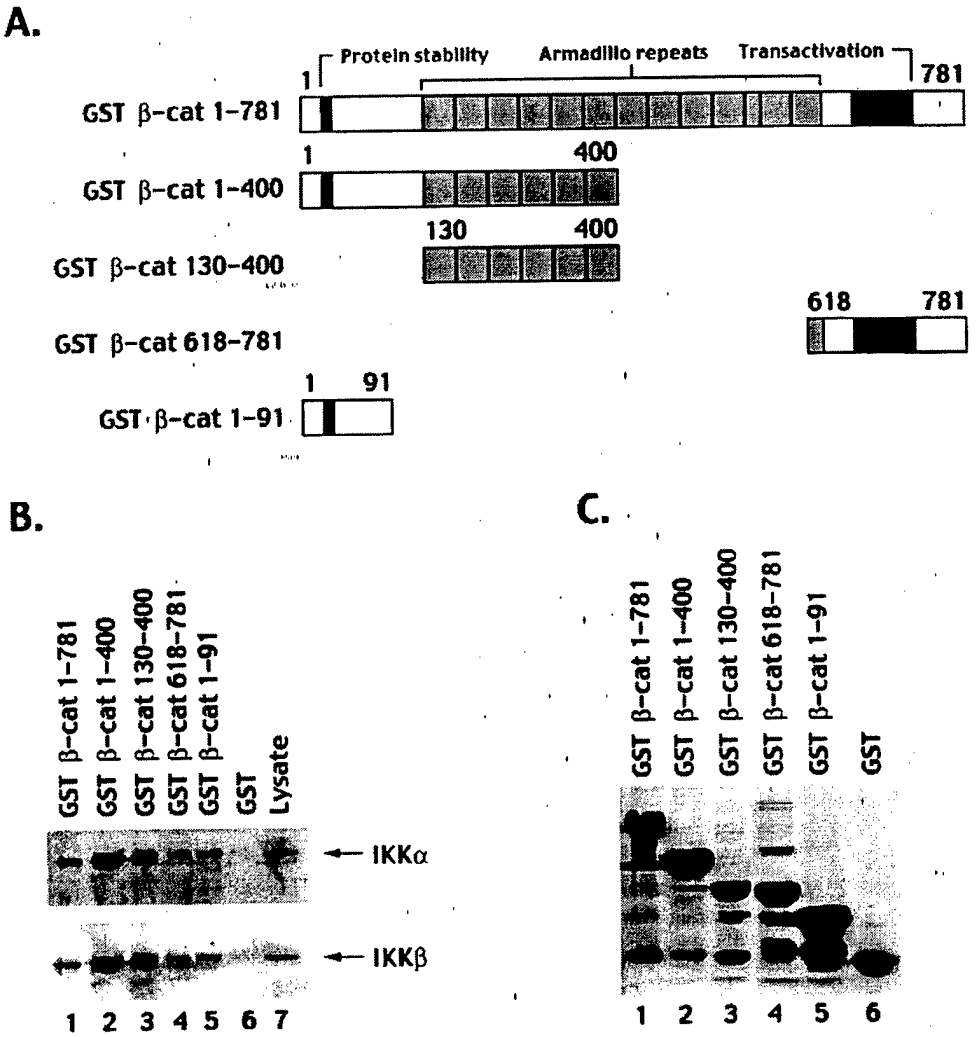


FIG. 6. **In vitro interaction of β-catenin with endogenous IKKα and IKKβ.** *A*, a schematic representation of the GST β-catenin fusion proteins that were used to analyze interactions with SW480 extract is shown. *B*, GST-fusion proteins with β-catenin were bound to glutathione-Sepharose beads (*lanes 1–6*), and after overnight incubation with SW480 cell extract, the glutathione beads were extensively washed and Western blot analysis was performed using rabbit polyclonal antibodies directed against either IKKα or IKKβ as indicated. 10% of the SW480 lysate alone is shown in *lane 7*. *C*, the GST-fusion proteins were analyzed by Coomassie Blue staining.

substrate was calculated (Fig. 8, *A* and *B*, *lower panels*).

Kinase assays performed with IKKα using 1 mM of cold ATP resulted in ~0.05 mol of phosphate/mol of protein incorporated into the amino terminus of β-catenin as compared with 0.09 mol of phosphate/mol of protein incorporated into the amino terminus of IκBα after a 120-min reaction (Fig. 8*A*). Kinase assays performed with IKKβ using 1 mM of cold ATP demonstrated that there was 1.4 mol of phosphate/mol of protein incorporated into the amino terminus of β-catenin and 0.5 mol of phosphate/mol of protein incorporated in the amino terminus of IκBα after 120 min (Fig. 8*B*). It is interesting to note that the phosphorylation of the β-catenin by IKKβ may be biphasic in contrast to its phosphorylation of IκBα (Fig. 8*B*). Similar phosphate incorporation into these substrates was found using both baculovirus-produced and COS-transfected IKKα and IKKβ proteins (data not shown). In agreement with previous studies, this analysis indicates that IKKα is a much weaker kinase than is IKKβ in phosphorylating IκBα (58) and β-catenin. These results indicate that the IKK proteins result in relatively similar incorporation of phosphate into the amino terminus of β-catenin and IκBα, although there are differences in the kinetics of this process.

DISCUSSION

In this study, we present data that IKKα and IKKβ can modulate β-catenin function. First, we observed the differential localization of β-catenin in mouse embryo fibroblasts derived from IKKα- and IKKβ-deficient cells. Second, the transcriptional activity of β-catenin was higher in IKKβ$^{-/-}$ cells as compared with IKKα$^{-/-}$ cells. Third, IKKβ decreased β-catenin-dependent gene expression similar to the effects seen with GSK-3β, while IKKα increased this activity. Fourth, we found that IKKα expression in COS cells increased the amount of β-catenin, while IKKβ expression reduced the amount of β-catenin. Finally, we demonstrated that IKKα and IKKβ interacted with and were able to phosphorylate β-catenin. Experiments are underway to map the sites in β-catenin that are phosphorylated by IKKα and IKKβ in order to determine whether phosphorylation alters β-catenin function. Our preliminary results suggest that IKKα phosphorylates different residues in the amino terminus of β-catenin than serine residues 33 and 37 that are phosphorylated by GSK-3β.

Studies with an amino-terminal deletion of β-catenin indicated that IKKα requires this region to increase β-catenin-de-

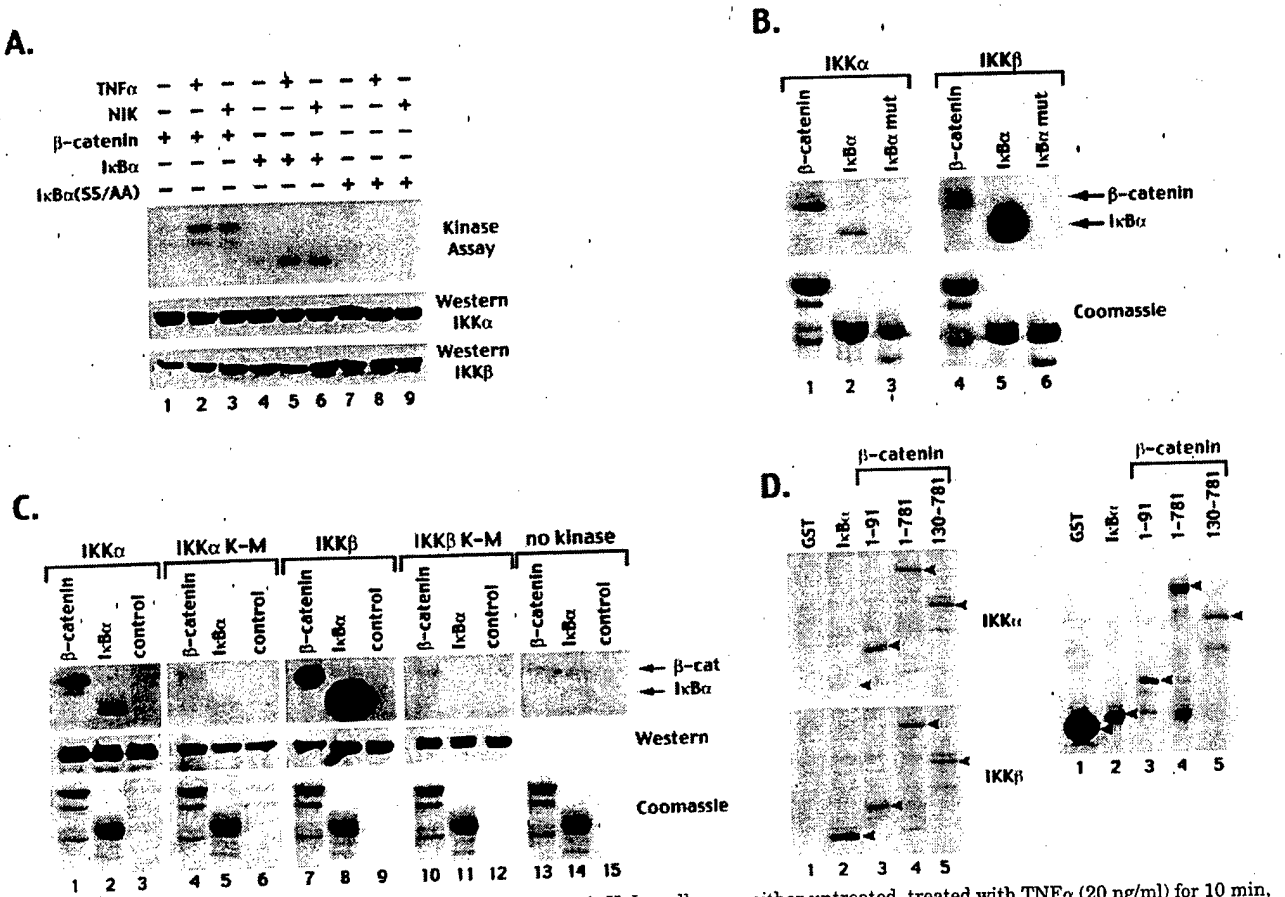


FIG. 7. **IKKα and IKKβ phosphorylate β-catenin and IκBα.** *A*, HeLa cells were either untreated, treated with TNFα (20 ng/ml) for 10 min, or transfected with a pCMV5 expression vector encoding NIK. Extracts were immunoprecipitated with a polyclonal antibody directed against IKKα and IKKβ and *in vitro* kinase assays were performed with GST fusions with β-catenin-(1–91), IκBα-(1–54) or IκBα SS/AA-(1–54) followed by autoradiography. *B*, recombinant baculovirus-expressed IKKα and IKKβ proteins were purified as described. *In vitro* kinase assays were performed using IKKα and IKKβ and GST-β-cat-(1–91) (*lanes 1* and *4*) and GST-IκBα-(1–54) (*lanes 2* and *5*) as substrates (*upper panel*). A GST-IκBα protein in which serine residues 32 and 36 were changed to alanine (*lanes 3* and *6*) was also assayed. The GST fusion proteins used in the *in vitro* kinase assay were monitored by Coomassie Blue staining (*lower panel*). *C*, COS cells were transfected with expression vectors encoding either FLAG-tagged wild-type or mutant IKKα or IKKβ kinases or mock transfected (*no kinase*). After 30 h, the cells were collected, and cellular extract was immunoprecipitated with the M2 monoclonal antibody directed against FLAG-epitope. The *upper panel* demonstrates *in vitro* phosphorylation of either GST-β-catenin (*lanes 1, 4, 7, 10,* and *13*), GST-IκBα (*lanes 2, 5, 8, 11,* and *14*) or no added substrate (*lanes 3, 6, 9, 12,* and *15*) by the indicated kinases. Expression of the transfected IKK constructs was analyzed by Western blot analysis (*middle panel*). Immunoprecipitation of extracts from mock-transfected cells were also analyzed in *in vitro* kinase assays (*lanes 13–15*). The amount of GST-β-catenin and GST-IκBα substrates used in these assays was monitored by Coomassie Blue staining (*lower panel*). *D*, FLAG-tagged IKKα (*top panel*) and IKKβ (*bottom panel*) immunoprecipitated from COS cell extracts were used in *in vitro* kinase assays with GST alone (*lane 1*) or GST fusions containing IκBα-(1–54) (*lane 2*), β-catenin-(1–91) (*lane 3*), β-catenin-(1–781) (*lane 4*), or β-catenin-(130–781) (*lane 5*). SDS-PAGE and autoradiography were then performed. A Coomassie-stained gel of the substrates used in the *in vitro* kinase assays is shown in the *right panel*.

pendent gene expression, while the effects of IKKβ on β-catenin activity are not dependent on this region. These results and the finding that IKKα is not able to increase the protein levels of an amino-terminal β-catenin mutant suggest that the amino terminus of β-catenin is likely involved in IKKα regulation. Thus, IKKα and IKKβ likely have effects on different domains of β-catenin to alter its role on gene expression. It is unclear whether differences in the kinase activity of IKKα and IKKβ are involved in their differential effects on β-catenin-dependent gene expression or whether other effects such as differential binding to specific pools of β-catenin may be involved. Finally, additional mechanisms such as IKK effects on β-catenin protein stability and/or nuclear import or export are possible. Multiple factors including Wnt signaling, the TCF/LEF proteins (19, 52, 53), and APC (59) affect the cellular localization of β-catenin, which lacks a canonical nuclear localization signal. Although we demonstrate that the IKK proteins interact with β-catenin, it is possible that IKK interaction with other components of the Wnt pathway such as APC may also be involved in regulating β-catenin function.

Both IKKα and IKKβ can form heterodimers and homodimers, and dimerization of these kinases is essential for their activity (33, 47, 60, 61). However, previous data has suggested that there is no synergy between IKKα and IKKβ in regulating their kinase activity (58). Given the wide disparity in their kinase activity, they may have other cellular targets in addition to IκB (58). The ability of these kinases to potentially associate with as yet unidentified cellular factors may alter their substrate specificity. Gene disruption studies indicate that IKKβ rather than IKKα is the critical kinase involved in the activation of the NF-κB pathway in response to treatment with either TNFα or IL-1β (37–39). The predominant cytoplasmic localization of IKKβ probably reflects the major role of this kinase in the phosphorylation of the IκB proteins that are localized in the cytoplasm bound to the RelA/p65 NF-κB protein (37–39). The results of our immunofluorescence studies

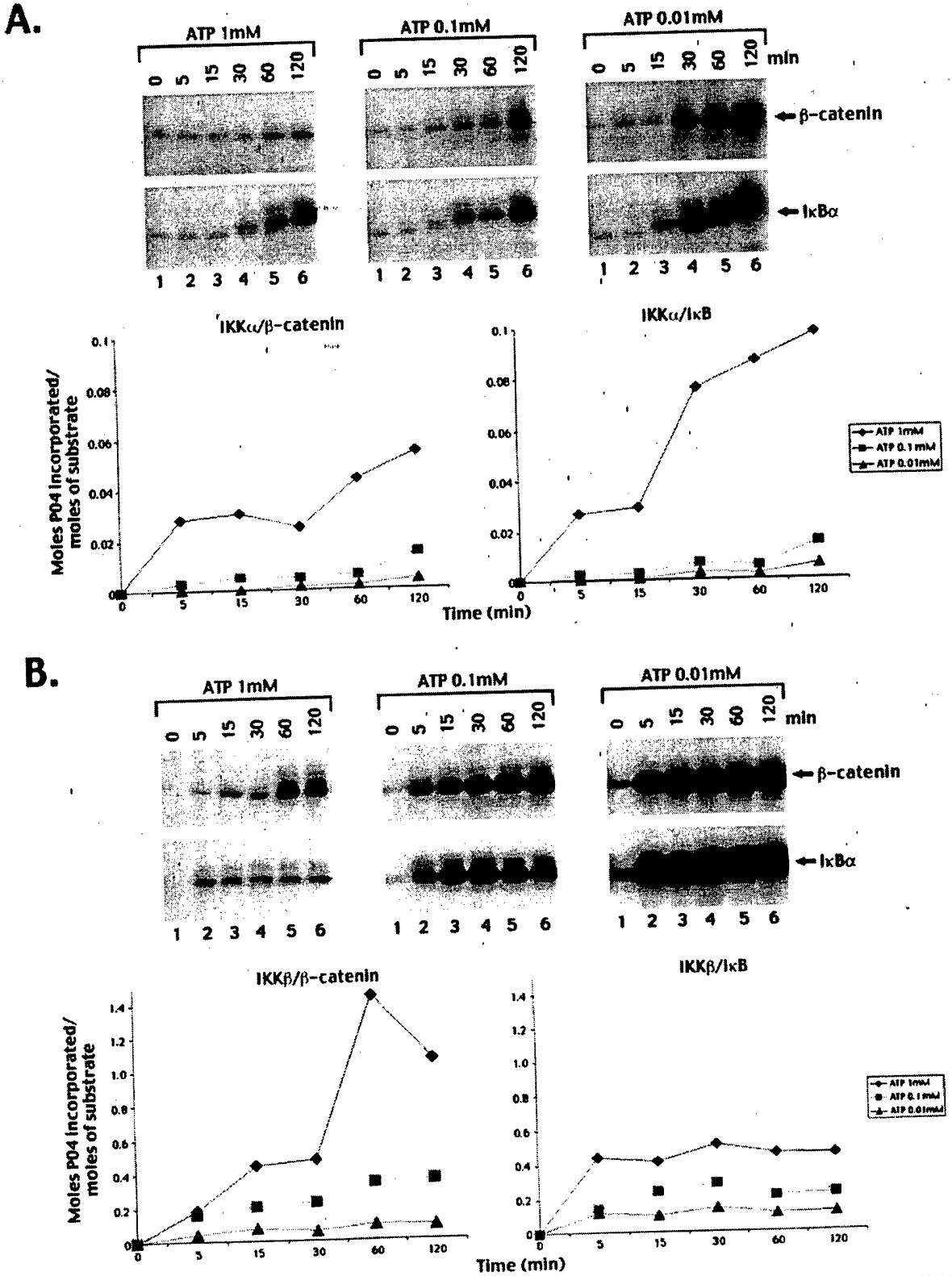


FIG. 8. **Stoichiometry of phosphate incorporation into the amino termini of IκBα and β-catenin by IKKα and IKKβ.** *A*, FLAG-tagged IKKα and *B*, IKKβ were immunoprecipitated from COS extracts with the M2 monoclonal antibody and incubated with 2 μg of GST fusions containing either β-catenin-(1–91) or IκBα-(1–54) in kinase buffer containing 15 μCi of [γ-^{32}P]ATP with a specific activity of 6000 Ci/mM and cold ATP at concentrations of 1 mM, 0.1 mM, and 0.01 mM. In vitro kinase reactions were performed for 0, 5, 15, 30, 60, and 120 min at 30 °C, and the samples were subjected to SDS-PAGE and autoradiography (*A* and *B*, *top panels*). Incorporation of ^{32}P into these substrates was quantitated by scintillation counting and the moles of phosphate incorporated per mole of substrate was calculated (*A* and *B*, *bottom panels*).

suggest that IKKα is localized in both the nucleus and cytoplasm of MEFs and may be predominantly nuclear in the absence of IKKβ in IKKβ$^{-/-}$ cells. Consistent with these observations, Western blot analysis of extracts prepared from COS cells transfected with expression vectors encoding IKKα and IKKβ indicate that IKKβ is predominantly localized in the cytoplasm, while IKKα is present in both the nucleus and the cytoplasm. Additional studies are currently underway to better characterize the cellular localization of IKKα. Whether any of the effects of IKKα on skin and skeletal development may in part be mediated by either IKKα binding and/or phosphorylation of β-catenin remains to be determined.

IKK regulation of β-catenin activity differs from its activation of the NF-κB pathway. Cytokines such as TNFα stimulate IKK phosphorylation of IκB leading to its rapid degradation and the nuclear translocation of NF-κB. TNFα activation of an NF-κB reporter construct is blocked by transfection of an IKKβ dominant negative mutant (30). Although TNFα treatment of cells results in marked decreases in β-catenin-dependent gene expression, this effect is only partially blocked by an IKKβ dominant negative mutant. These results suggest that the effects of TNFα on β-catenin-dependent gene expression likely involve additional substrates and/or pathways other than IKKβ and β-catenin. Although our results support a role for IKKα and IKKβ on modulating β-catenin activity, the regulation of this pathway is different from that seen with TNFα-induction of IKK to activate the NF-κB pathway.

Several observations are also consistent with the potential for similar factors being involved in the regulation of the Wnt and NF-κB pathways. It has been demonstrated that β-catenin/TCF signaling increases β-TrCP levels by a posttranscriptional mechanism to result in increased degradation of both β-catenin and IκB (45). Thus, changes of β-TrCP levels can result in marked effects on both the β-catenin and NF-κB pathways. Additionally, GSK-3β, which is an important kinase involved in regulating β-catenin levels, has also been implicated in regulating NF-κβ activation. Gene disruption studies have indicated that GSK-3β$^{-/-}$ mice have a phenotype similar to IKKβ-deficient mice developing liver degeneration as a result of increased sensitivity to TNFα stimulation (46). The mechanism by which GSK-3β may alter the NF-κB pathway remains to be determined. In summary, our studies suggest that a common set of cellular factors may be involved in the integration of a variety of cellular signaling processes that regulate the NF-κB and β-catenin pathways.

Acknowledgments—We thank Ken Kinzler for providing β-catenin and the TOPFLASH reporter constructs, Rudolf Grosschedl for providing the expression vector encoding LEF-1, Masahiro Aoki for providing additional LEF constructs, and Xiaodong Wang for providing the MEF cells. We also thank Melanie Cobb for helpful suggestions and Sharon Johnson and Alex Herrera for preparation of the manuscript and figures, respectively.

REFERENCES

1. Kemler, R. (1993) *Trends Genet.* **9,** 317–321
2. Ben-Ze'ev, A., & Geiger, B. (1998) *Curr. Opin. Cell Biol.* **10,** 629–639
3. Peifer, M., & Wieschaus, E. (1990) *Cell* **63,** 1167–1176
4. McCrea, P. D., Brieher, W. M., & Gumbiner, B. M. (1993) *J. Cell Biol.* **123,** 477–484
5. Funayama, N., Fagotto, F., McCrea, P., & Gumbiner, B. M. (1995) *J. Cell Biol.* **128,** 959–968
6. Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y., & Wylie, C. (1994) *Cell* **79,** 791–803
7. Laurent, M. N., Blitz, I. L., Hashimoto, C., Rothbacher, U., & Cho, K. W. (1997) *Development* **124,** 4905–4916
8. Morin, P. J. (1999) *Bioessays* **21,** 1021–1030
9. Polakis, P. (2000) *Genes Dev.* **14,** 1837–1851
10. Laney, J. D., & Hochstrasser, M. (1999) *Cell* **97,** 427–430
11. Maniatis, T. (1999) *Genes Dev.* **13,** 505–510
12. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., & Polakis, P. (1993) *Science* **262,** 1731–1734
13. Su, L. K., Vogelstein, B., & Kinzler, K. W. (1993) *Science* **262,** 1734–1737
14. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., & Polakis, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3046–3050
15. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., & Clevers, H. (1997) *Science* **275,** 1784–1787
16. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., & Kinzler, K. W. (1997) *Science* **275,** 1787–1790
17. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., & Polakis, P. (1997) *Science* **275,** 1790–1792
18. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., & Birchmeier, W. (1996) *Nature* **382,** 638–642
19. Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., & Kemler, R. (1996) *Mech. Dev.* **59,** 3–10
20. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., & Clevers, H. (1996) *Cell* **86,** 391–399
21. Brunner, E., Peter, O., Schweizer, L. ,& Basler, K. (1997) *Nature* **385,** 829–833
22. Giese, K., & Grosschedl, R. (1993) *EMBO J.* **12,** 4667–4676
23. Giese, K., Kingsley, C., Kirshner, J. R., & Grosschedl, R. (1995) *Genes Dev.* **9,** 995–1008
24. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., & Kinzler, K. W. (1998) *Science* **281,** 1509–1512
25. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., & Ben-Ze'ev, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5522–5527
26. Tetsu, O., & McCormick, F. (1999) *Nature* **398,** 422–426
27. Baldwin, A. S. (1996) *Annu. Rev. Immunol.* **14,** 649–681
28. Ghosh, S., May, M. J., & Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
29. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., & Karin, M. (1997) *Nature* **388,** 548–554
30. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., & Mann, M. (1997) *Science* **278,** 860–866
31. Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., & Rothe, M. (1997) *Cell* **90,** 373–383
32. Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., & Goeddel, D. V. (1997) *Science* **278,** 866–869
33. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., & Karin, M. (1997) *Cell* **91,** 243–252
34. Rothwarf, D. M., Zandi, E., Natoli, G., & Karin, M. (1998) *Nature* **395,** 297–300
35. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., & Israel, A. (1998) *Cell* **93,** 1231–1240
36. Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Pascual, G., Motiwala, A., Zhu, H., Mann, M., & Manning, A. M. (1999) *Mol. Cell. Biol.* **19,** 1526–1538
37. Li, Z. W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., & Karin, M. (1999) *J. Exp. Med.* **189,** 1839–1845
38. Tanaka, M., Fuentes, M. E., Yamaguchi, K., Durnin, M. H., Dalrymple, S. A., Hardy, K. L., & Goeddel, D. V. (1999) *Immunity* **10,** 421–429
39. Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F., & Verma, I. M. (1999) *Science* **284,** 321–325
40. Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., & Karin, M. (1999) *Science* **284,** 316–320
41. Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., & Akira, S. (1999) *Science* **284,** 313–316
42. Li, Q., Lu, Q., Hwang, J. Y., Buscher, D., Lee, K. F., Izpisua-Belmonte, J. C., & Verma, I. M. (1999) *Genes Dev.* **13,** 1322–1328
43. Karin, M., & Ben-Neriah, Y. (2000) *Annu Rev of Immunol.* **18,** 621–663
44. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C., Elledge, S. J., & Harper, J. W. (1999) *Genes Dev.* **13,** 270–283
45. Spiegelman, V. S., Slaga, T. J., Pagano, M., Minamoto, T., Ronai, Z., & Fuchs, S. Y. (2000) *Mol. Cell* **5,** 877–882
46. Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O., & Woodgett, J. R. (2000) *Nature* **406,** 86–90
47. Kwak, Y. T., Guo, J., Shen, J., & Gaynor, R. B. (2000) *J. Biol. Chem.* **275,** 14752–14759
48. Yamamoto, Y., Yin, M. J., & Gaynor, R. B. (2000) *Mol. Cell. Biol.* **20,** 3655–3666
49. Li, X.-H., Murphy, K. M., Palka, K. T., Surabhi, R. M., & Gaynor, R. B. (1999) *J. Biol. Chem.* **274,** 34417–34424
50. Munemitsu, S., Albert, I., Rubinfeld, B., & Polakis, P. (1996) *Mol. Cell. Biol.* **16,** 4088–4094
51. Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., & Moon, R. T. (1996) *Genes Dev.* **10,** 1443–1454
52. Pai, L. M., Orsulic, S., Bejsovec, A. & Peifer, M. (1997) *Development* **124,** 2255–2266,
53. Hsu, S. C., Galceran, J., & Grosschedl, R. (1998) *Mol. Cell. Biol.* **18,** 4807–4818
54. Wong, M. H., Rubinfeld, B., & Gordon, J. I. (1998) *J. Cell Biol.* **141,** 765–777
55. Chen, Z. J., Parent, L., & Maniatis, T. (1996) *Cell* **84,** 853–862
56. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., & Wallach, D. (1997) *Nature* **385,** 540–548
57. Nemoto, S., DiDonato, J. A., & Lin, A. (1998) *Mol. Cell. Biol.* **18,** 7336–7343
58. Li, J., Peet, G. W., Pullen, S. S., Schembri-King, J., Warren, T. C., Marcu, K. B., Kehry, M. R., Barton, R., & Jakes, S. (1998) *J. Biol. Chem.* **273,** 30736–30741
59. Rosin-Arbesfeld, R., Townsley, F., & Blenz, M. (2000) *Nature* **406,** 1009–1012
60. Zandi, E., Chen, Y., & Karin, M. (1998) *Science* **281,** 1360–1363
61. Delhase, M., Hayakawa, M., Chen, Y., & Karin, M. (1999) *Science* **284,** 309–313

Molecular Biology of the Cell
Vol. 14, 585–599, February 2003

IKKα Regulates Mitogenic Signaling through Transcriptional Induction of Cyclin D1 via Tcf

Chris Albanese,*¶# Kongming Wu,*¶# Mark D'Amico,*# Christy Jarrett,† David Joyce,* Julian Hughes,* James Hulit,* Toshiyuki Sakamaki,*# Maofu Fu,*# Avri Ben-Ze'ev,‡ Jacqueline F. Bromberg,§ Carmela Lamberti,‖ Udit Verma,‖ Richard B. Gaynor,‖ Stephen W. Byers,† and Richard G. Pestell*#@

*The Albert Einstein Cancer Center, Division of Hormone-Dependent Tumor Biology, The Albert Einstein Comprehensive Cancer Center, Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461; †Department of Oncology, Lombardi Cancer Center and Department of Cell Biology; Georgetown University School of Medicine, Washington, DC 20007; ‡Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel; §Department of Medicine, Memorial Sloan–Kettering Cancer Center, New York, New York 10021; and ‖Division of Hematology-Oncology, Department of Medicine, Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Submitted June 28, 2002; Revised August 27, 2002; Accepted October 16, 2002
Monitoring Editor: Pamela A. Silver

The Wnt/β-catenin/Tcf and IκB/NF-κB cascades are independent pathways involved in cell cycle control, cellular differentiation, and inflammation. Constitutive Wnt/β-catenin signaling occurs in certain cancers from mutation of components of the pathway and from activating growth factor receptors, including RON and MET. The resulting accumulation of cytoplasmic and nuclear β-catenin interacts with the Tcf/LEF transcription factors to induce target genes. The IκB kinase complex (IKK) that phosphorylates IκB contains IKKα, IKKβ, and IKKγ. Here we show that the *cyclin D1* gene functions as a point of convergence between the Wnt/β-catenin and IκB pathways in mitogenic signaling. Mitogenic induction of G_1-S phase progression and cyclin D1 expression was PI3K dependent, and *cyclin D1*$^{-/-}$ cells showed reduced PI3K-dependent S-phase entry. PI3K-dependent induction of cyclin D1 was blocked by inhibitors of PI3K/Akt/IκB/IKKα or β-catenin signaling. A single Tcf site in the cyclin D1 promoter was required for induction by PI3K or IKKα. In IKKα$^{-/-}$ cells, mitogen-induced DNA synthesis, and expression of Tcf-responsive genes was reduced. Reintroduction of IKKα restored normal mitogen induction of cyclin D1 through a Tcf site. In IKKα$^{-/-}$ cells, β-catenin phosphorylation was decreased and purified IKKα was sufficient for phosphorylation of β-catenin through its N-terminus in vitro. Because IKKα but not IKKβ induced cyclin D1 expression through Tcf activity, these studies indicate that the relative levels of IKKα and IKKβ may alter their substrate and signaling specificities to regulate mitogen-induced DNA synthesis through distinct mechanisms.

INTRODUCTION

The Wingless/Wnt pathway plays a crucial role in development and cell cycle control (Cadigan and Nusse, 1997; Huelsken and Behrens, 2000). Dysregulation of the Wingless/(Wnt)/β-catenin/Tcf pathway has been implicated in tumorigenesis of diverse types (Polakis, 2000a). Axin/Conductin, together with APC, promote β-catenin degradation through serine-threonine phosphorylation of the β-catenin N-terminus by GSK3β, which targets β-catenin for ubiquitination by a $SCF^{\beta\text{-}TRCP}$ (β-transducin repeat-containing protein) ubiquitin ligase complex (Fuchs *et al.*, 1999; Winston *et al.*, 1999) and its degradation by the proteasome. On induction of Wnt signaling by extracellular ligands, the Frizzled receptors are activated. The activity of GSK3β and its effect on β-catenin is antagonized by Dishevelled, a downstream target of Frizzled, thus preventing the degradation of β-cate-

DOI: 10.1091/mbc.02–06–0101.
@ Corresponding author. E-mail address: pestell@georgetown.edu.
¶ Both authors contributed equally to this work.
# Present addresses: Department of Oncology, Lombardi Cancer Center, Georgetown University School of Medicine, Washington, D.C. 20007.

nin by the proteasome. The resulting accumulation of β-catenin leads to its nuclear translocation and binding to Tcf/Lef transcription factors to induce target genes including *cyclin D1* and *c-Myc* (He *et al.*, 1998; Shtutman *et al.*, 1999; Huelsken and Behrens, 2000).

In addition to components in the Wnt signaling pathway, several other pathways can regulate β-catenin/Tcf signaling and gene expression and confer aberrant cellular growth. The protein encoded by *Gas6*, a growth factor of the vitamin K–dependent family, which binds members of the Axl receptor tyrosine kinase family, stabilizes β-catenin, and induces Tcf signaling (Goruppi *et al.*, 2001). Hepatocyte growth factor/scatter factor (Papkoff and Aikawa, 1998) and oncogenic mutations of RON and MET (Danilkovitch-Miagkova *et al.*, 2001) can also increase cytosolic β-catenin and activate Lef/Tcf-responsive reporters. The *Xenopus* wnt target gene *twin* is induced by SMAD4 through the β-catenin/Tcf complex (Nishita *et al.*, 2000). Conversely, genotoxic stress reduces β-catenin abundance in part through p53 signaling and a Siah1/Skp1/Ebi complex, which binds the β-catenin N-terminus independently of its GSK3β phosphorylation sites (Liu *et al.*, 2001; Matsuzawa and Reed, 2001).

The *c-myc* and *cyclin D1* genes that encode important regulators of cell proliferation have been identified as transcriptional targets of β-catenin (He *et al.*, 1998; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999). Transcription of the *cyclin D1* gene is induced through distinct DNA sequences in the promoter by diverse mitogenic and oncogenic signaling pathways including activating mutants of Ras, Src, Stat3, Stat5, and ErbB-2 (Albanese *et al.*, 1995; Bromberg *et al.*, 1999; Matsumura *et al.*, 1999; Pestell *et al.*, 1999; Lee *et al.*, 2000). Distinct binding sites within the cyclin D1 promoter have been characterized for transcription factors including CREB and AP-1 proteins (Albanese *et al.*, 1995; Watanabe *et al.*, 1996a, 1996b; Brown *et al.*, 1998), and a single site at −81 has shown to bind β-catenin/Tcf proteins (Shtutman *et al.*, 1999). Although Tcf/Lef proteins can function as either enhancer or repressor elements (Bienz, 1998; Barker *et al.*, 2000), the Tcf binding site of the cyclin D1 promoter at −81 functioned as an enhancer element that conveyed activation of the cyclin D1 promoter by components of the Wnt/β–catenin pathway (Shtutman *et al.*, 1999; D'Amico *et al.*, 2000; Lin *et al.*, 2000; Sampson *et al.*, 2001; Soriano *et al.*, 2001). The *cyclin D1* gene encodes a regulatory subunit of the holoenzyme that phosphorylates and inactivates the retinoblastoma (pRB) protein. Homozygous deletion of the *cyclin D1* gene in mice demonstrated a requirement for cyclin D1 in normal mammary gland development during pregnancy and mouse embryo fibroblasts (MEFs) derived from the *cyclin D1*$^{-/-}$ animals have both defective induction of DNA synthesis and enhanced cellular apoptosis rates (Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Albanese *et al.*, 1999; Fantl *et al.*, 1999). Cyclin D1 overexpression can enhance DNA synthesis, is required for transformation and contact-independent growth in several cell types and has been implicated in several human cancers including breast, colon, and prostate (Shtutman *et al.*, 1999; Tetsu and McCormick, 1999; Lee *et al.*, 2000). Thus, cyclin D1 plays an important role in tumorigenesis and cell cycle control.

The IκB/NF-κB pathway is another pathway involved in both cell cycle control and inflammation and has recently been implicated in cancer (Karin and Delhase, 2000; Yamamoto and Gaynor, 2001). The NF-κB transcriptional activity is normally inhibited by IκB proteins that sequester it in the cytoplasm (Karin and Delhase, 2000; Joyce *et al.*, 2001). The IκB kinase complex (IKK) that phosphorylates IκB contains two functionally distinct kinases, IKKα and IKKβ. IKKβ plays a dominant role in NF-κB regulation by TNF-α and IL-1 (Delhase *et al.*, 1999; Li *et al.*, 1999a). In contrast, IKKα is required for murine skeletal and keratinocyte differentiation (Li *et al.*, 1999a; Takeda *et al.*, 1999; Hu *et al.*, 2001). IKKα cannot compensate for the loss of IKKβ (Li *et al.*, 1999a), suggesting that distinct targets are regulated by IKKα and IKKβ. Although the IκB/NF-κB and Wnt/β-catenin/Tcf pathways are independent signaling pathways, both IκB and β-catenin are regulated by phosphorylation at similar consensus N-terminal serines and are targeted for ubiquitination by a similar $SCF^{\beta\text{-}TrCP}$ complex followed by proteasomal degradation. The consequences of this regulation are, however, very different (Fuchs *et al.*, 1999; Winston *et al.*, 1999). Thus, although the $SCF^{\beta\text{-}TrCP}$-mediated degradation of IκB leads to the induction of NF-κB activity, the $SCF^{\beta\text{-}TrCP}$-mediated degradation of β-catenin inhibits the activity of the Wnt pathway. In addition, although GSK3β contributes to the degradation of β-catenin and represses β-catenin/Tcf signaling, the activity of NF-κB is enhanced by GSK3β (Hoeflich *et al.*, 2000; Polakis, 2000a).

The IKK complex is regulated by several IKK kinases including the NF-κB inducing kinase (NIK), TAK1, MEKK1, Cot/TPL2, and NAK, which coordinate physiological responses to distinct stimuli (Joyce *et al.*, 2001). NF-κB activity is also enhanced by the serine threonine kinase Akt (Madrid *et al.*, 2000; Romashkova and Makarov, 1999) that is known to induce cellular proliferation and survival (Datta *et al.*, 1999) in response to PI3K activation (Franke *et al.*, 1997; Klippel *et al.*, 1998). Akt is recruited to IKKα by stimulation with growth factors, but not by TNF-α. Akt activation by PI3K is inhibited by the tumor suppressor PTEN, a D3 phosphoinositide phosphatase that induces G_1 arrest in prostate cancer cells (Ramaswamy *et al.*, 1999), consistent with both a role for PTEN as a prostate cancer cell tumor suppressor and a role of PI3K-Akt activation in cell cycle progression (Di Cristofano *et al.*, 2001). The Gas6-dependent proliferation and activation of Tcf is also dependent on PI3K (Goruppi *et al.*, 2001), suggesting a role for PI3K signaling in the regulation of β-catenin/Tcf signaling. The components of the cell cycle machinery that are regulated by IKKα and are required for PI3K-dependent cellular proliferation, however, remain to be determined.

Here we show a novel role for IKKα in mitogenic signaling through transcriptional induction of the *cyclin D1* gene. We show that the serum induction of cyclin D1 and G_1-S phase progression is PI3K-dependent and that cells lacking cyclin D1 show a reduction in PI3K-dependent S-phase entry. PI3K-dependent induction of cyclin D1 was blocked by an inhibitor of IKKα and activation of IKKα-induced cyclin D1. PI3K induction of cyclin D1 was inhibited by a dominant negative Tcf, and a single Tcf site in the cyclin D1 promoter was required for its induction by IKKα and PI3K. Mouse embryo fibroblasts derived from mice lacking IKKα showed reduced phosphorylation of β-catenin and reduced Tcf and cyclin D1 abundance and promoter activity. We had previously shown that IKKα exists in a complex with endogenous

β-catenin (Lamberti *et al.*, 2001). Herein we show that purified IKKα was sufficient for phosphorylation of β-catenin through its N-terminus in vitro, demonstrating that IKKα can function as a kinase independently of its heterodimeric partners. Because IKKα but not IKKβ induced cyclin D1 expresion and Tcf activity, these studies indicate that the relative levels of IKKα and IKKβ may alter their substrate and signaling specificities to regulate DNA synthesis through distinct mechanisms.

MATERIALS AND METHODS

Construction of Reporter Genes and Expression Vectors

The human cyclin D1 promoter fragments linked to the luciferase reporter gene in the pA_3LUC vector promoters of the c-*fos* gene (c-*fos*LUC), TOP-FLASH, FOP-FLASH, cyclinELUC, cyclinALUC, c-*Myc*LUC, Engrailed 2 promoter (*Engr*LUC), 3xRelLUC, and pGL_3LUC (Promega, Madison, WI) were previously described (He *et al.*, 1998; Joyce *et al.*, 1999; McGrew *et al.*, 1999; D'Amico *et al.*, 2000; Lee *et al.*, 2000). The expression vectors for p110-K227E, p110-CAAX (Matsumura *et al.*, 1999), the p110-kinase dead, the p85α, p85ΔiSH2-N, 85ΔiSH2-C, p85ΔbBCR were kind gifts from Dr. J. Downward (Rodriguez-Viciana *et al.*, 1997); pCMV-c-Akt wt, Akt-K179 M, Akt-T308A, were from Dr. A. Bellacosa; and CMV-IκB (Super-repressor) [CMV-IκBα (Sr)] was a gift from Dr. D. Ballard (Brockman *et al.*, 1995). Mammalian expression vectors for IKKα (S176/180E and A) and IKKβ (S177/181E and A) mutants were provided by Dr. F. Mercurio and for IKKα (K54 M) and IKKβ (K44A) were provided by Tularik Inc (South San Francisco, CA).

Reporter Assays, Cell Culture, and Chemicals

Cell culture and DNA transfection were performed exactly as previously described (Lipofectamine Plus; Life Technologies BRL, Rockville, MD; DiDonato *et al.*, 1997; Zandi *et al.*, 1997). Transfections were normalized using RSV-β-gal unless otherwise indicated (DiDonato *et al.*, 1997; Zandi *et al.*, 1997). The effect of an expression vector was compared with the effect of an equal amount of vector cassette. The DU145 cells were maintained in DMEM with 10% (vol/vol) calf serum and 1% penicillin/streptomycin. SW480 colon cancer cells and Cos-7 kidney cells were grown in DMEM (5% fetal bovine serum). The *IKKα*$^{-/-}$ mouse embryo fibroblasts (MEFS) and 3T3 cells were a generous gift from Dr. M. Karin. Cells were plated at ~100,000 cells/well in 12-well plates. After 24 h, cells were transfected with the indicated DNA and a Renilla luciferase reporter as an internal control for transfection efficiency. All transfections were done at least in triplicate and were repeated at least three times. Treatments with the PI 3-kinase inhibitor LY294002, the MEK inhibitor PD098059 (10–20 μM), the p38 MAP kinase inhibitor SB203580 (10–20 μM), wortmannin (2, 5, 10 μM) were performed for 24 h, and results were compared with vehicle treatment. Luciferase assays were performed at room temperature using an AutoLumat LB 953 (EG&G Berthold, Natick, MA). Luciferase content was measured by calculating the light emitted during the initial 10 s of the reaction, and the values are expressed in arbitrary light units. Statistical analyses were performed using the Mann Whitney *U* test with significant differences established as $p < 0.05$. To select transfected cells, cotransfection experiments were conducted using magnetic separation of transfected cells using CD4 as the marker and the magnetic-activated cell separation system (MACS; Ashton *et al.*, 1999).

Western Blots and Cell Cycle Analysis

Western blotting was performed with antibodies directed to cyclin D1 (DCS-6; NeoMarkers, Fremont, CA), TFIIB (Transduction Laboratories, Lexington, KY), IKKα (mAb was from PharMingen, San Diego, CA), IKKα, (polyclonal SC7182, Santa Cruz Biotechnology, Santa Cruz, CA) IKKβ, (polyclonal SC7607, Santa Cruz Biotechnology), β-catenin (Transduction Laboratories), phospho-β-catenin (Cell Signaling, Beverly, MA), Flag, (M2, Sigma Chemical Co., St. Louis, MO) and HA (12CA5, Sigma). Cell homogenates (50 μg) were electrophoresed in an SDS-12% polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane (Micron Separations Inc., Westborough, MA). After transfer, the gel was stained with Coomassie blue as a control for blotting efficiency. The blotting membrane was incubated for 2 h at 25°C in T-PBS buffer supplemented with 5% (wt/vol) dry milk to block nonspecific binding sites. After a 6-h incubation with primary antibody at a 1:1000 dilution (cyclin D1) or 1:2500 (α-tubulin) in T-PBS buffer containing 0.05% (vol/vol) Tween 20, the membrane was washed with the same buffer. For detection of cyclin D1 the membrane was incubated with goat anti-mouse horseradish peroxidase second antibody (Santa Cruz Biotechnology) and washed again. Immunoreactive proteins were visualized by the enhanced chemiluminescence system (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Annexin V staining for apoptosis (Albanese *et al.*, 1999) and cell cycle analysis were performed by flow cytometric analyses using a fluorescence-activated cell sorter (FACStar plus; Becton Dickinson & Co., Lincoln Park, NJ).

In Vitro Kinase Assays

Kinase assays were performed as described (Yamamoto *et al.*, 2000). The baculovirus-produced IKKα protein was purified by nickel-agarose chromatography and then immunoprecipitated with 12CA5 mAb (Yamamoto *et al.*, 2000). IKKα was added to kinase buffer containing 10 μCi of [γ-^{32}P], 1 mM ATP, 1 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, and then 1 μg of each of the substrates including GST-IκBα (1–54) or GST-β-cat constructs (Lamberti *et al.*, 2001) was incubated for 15 min at 30°C. Reactions were incubated at 30°C for 30 min and stopped by the addition of protein loading buffer and heating to 90°C and SDS-PAGE and autoradiography.

RESULTS

PI3K-induction of Cyclin D1 Requires the Tcf Binding Site

Activation of phosphatidyl inositol 3′-kinase (PI3K) mediates signaling induced by a number of growth factors and tumor promoters and is required for mitogenic stimulation by specific growth factors during the G_1-S phase of the cell cycle (Klippel *et al.*, 1998; Vanhaesebroeck and Waterfield, 1999). The role of PI3K in serum-induced cyclin D1 expression was examined in mouse embryo fibroblasts (MEFs). In wild-type (wt) MEFs, cyclin D1 protein levels were elevated by 3 h after serum stimulation, and the PI3K inhibitor LY294002 abrogated the induction (Figure 1A). Total ERK levels were unchanged under these conditions in both wt and *Cyclin D1*$^{-/-}$ MEFs (Figure 1A). Activity of the full-length human cyclin D1 promoter linked to a luciferase reporter gene was induced 2.5-fold by serum addition. The PI3K inhibitor reduced serum-induced activation of the cyclin D1 promoter by 80% (Figure 1B). Activation of PI3K and Akt plays a key role in DNA synthesis in prostate cancer cells (Ramaswamy *et al.*, 1999; Di Cristofano *et al.*, 2001). We therefore examined the role of PI3K in the PTEN containing prostate cancer cell line DU145. Because PI3K plays a role in signaling by diverse growth factors, including Gas6 in density-arrested cells (Goruppi *et al.*, 2001), we examined the

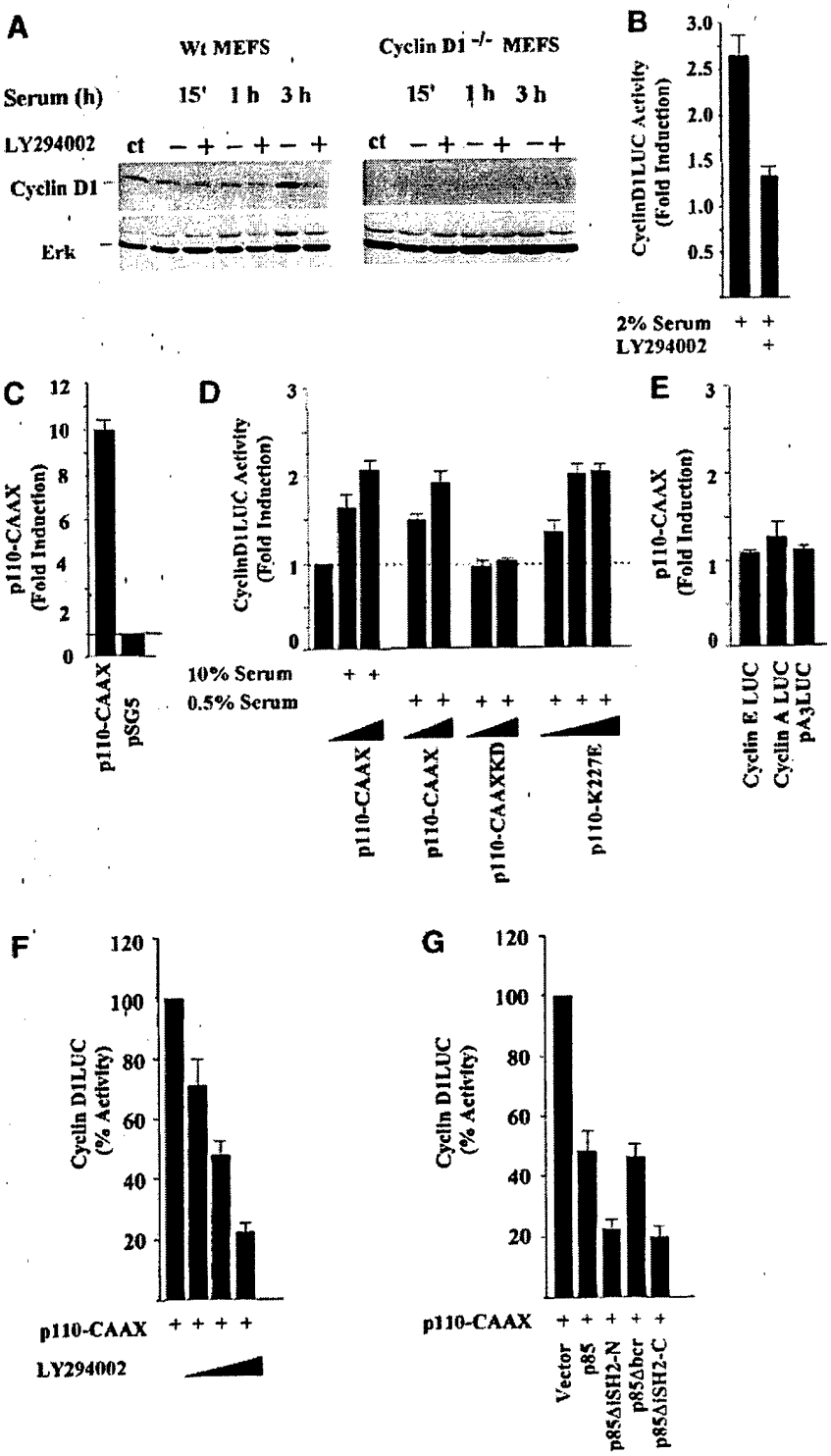


Figure 1. PI3K-induction of cyclin D1. (A) Western blot analysis of MEFs for cyclin D1 derived from wild-type (*cyclin D1* wt) or *cyclin D1*$^{-/-}$ mice and treated with serum either with or without the PI3K inhibitor LY294002. The Western blot was probed for cyclin D1 and total ERK. (B) The serum-induced activity of the cyclin D1 promoter in the presence or absence of the PI3 kinases inhibitor LY294002 (20 μM). (C) DU145 cells at either >90% or (D) 30% confluence were transfected with a cyclin D1 promoter luciferase reporter plasmid (−1745CD1LUC) and either the p110α-CAAX, or (D) the p110α kinase dead mutant (p110α-CAAX-KD) or the constitutively active p110α-K227E mutant expression plasmid in the presence of either 10% or 0.5% serum. The fold induction of the luciferase reporter activity is shown for nine separate experiments as mean ± SEM throughout. (E) The effect of p110-CAAX on reporter plasmids for cyclin A and the cyclin E promoter, and the luciferase reporter pA$_3$LUC. (F) The p110α-CAAX induction of the cyclin D1 promoter activity was inhibited by LY294002 (using 2, 20, and 100 μM). (G) The cyclin D1 promoter activity in the presence of p110α-CAAX is shown as 100% and is compared with the effect of cotransfected dominant negative inhibitors of PI3K including p85α, p85ΔiSH2-N, 85ΔiSH2-C, or p85ΔBCR (Rodriguez-Viciana *et al.*, 1997). The results are shown compared with equal amounts of empty control vector for each expression vector plasmid.

regulation of cyclin D1 by PI3K in density-arrested cells. The cyclin D1 promoter (−1745 CD1LUC) was induced 10-fold by p110α-CAAX compared with the empty vector (Figure 1C). In low-confluence cells the cyclin D1 promoter was induced significantly by p110α-CAAX in either high (Figure 1C) or low serum conditions (2.3-fold ± 0.18, n = 11, p <

0.01; Figure 1D). The kinase dead mutant (p110α-CAAX KD) did not affect cyclin D1 promoter activity, and the constitutively active p110α-K227E mutant induced cyclin D1 2.2-fold (Figure 1D). In contrast with the cyclin D1 promoter, the cyclin E and cyclin A promoters were not induced by p110α-CAAX (Figure 1E), suggesting that the induction of cyclin D1 is not an indirect effect of PI3K activity on DNA synthesis and the effect of p110α-CAAX is promoter specific. Because cryptic activation sequences, including AP-1, have been identified in several expression vectors, we examined the empty luciferase reporter pA_3LUC in which the cyclin D1 promoter was cloned and found that pA_3LUC was not induced (Figure 1E) in contrast with pGL_3LUC, which was induced threefold by p110α-CAAX (Amanatullah *et al.*, 2001). Cyclin D1 promoter activation by PI3K was reduced by the chemical inhibitor LY294002 (Figure 1F) or Wortmannin (our unpublished results). Type 1 PI3K is a heterodimeric holoenzyme, consisting of a regulatory (p85) and a catalytic (p110) subunit, which was initially identified through its role in Src-mediated transformation. p110α-CAAX induction of cyclin D1 promoter activity was reduced by the previously described dominant inhibitory mutants of the PI3K regulatory subunit (Rodriguez-Viciana *et al.*, 1997; Figure 1G).

Oncogenic forms of p110α and p85 have been identified, and expression of a constitutively active PI3K was shown to trigger DNA synthesis through activation of several distinct signaling pathways (Chang *et al.*, 1997; Klippel *et al.*, 1998). The cyclin D1 promoter contains several distinct transcription factors binding sites targeted by different signaling pathways (reviewed in Pestell *et al.*, 1999). Using a series of 5′ cyclin D1 promoter deletion constructions, the minimal p110α-CAAX responsive region was identified within the proximal 163 base pairs, which includes a Tcf site at −81 (our unpublished results). Point mutation of this sequence in the context of the −1745-base pair promoter fragment abolished induction at either high confluence (Figure 2A) or at low confluence (Figure 2B). p110α-CAAX induced the Tcf response element (TOP-FLASH) but had no effect on a reporter construct in which the Tcf site fails to bind Tcf/β-catenin (FOP-FLASH; Figure 2B). A constitutively active stable mutant of β-catenin (β-catenin Y33), found in colon cancer and the SW48 colon cancer cell line, activates β-catenin signaling when transfected into cultured cells. The sequence of the cyclin D1 promoter Tcf site is identical to the canonical sequence of the TOP-FLASH reporter. Consistent with the identification of a single Tcf site in the cyclin D1 promoter required for regulation by β-catenin/Tcf signaling in several studies (Shtutman *et al.*, 1999; Lin *et al.*, 2000; Soriano *et al.*, 2001), the cyclin D1 promoter was induced twofold by β-catenin Y33 in DU145 ($p < 0.01$, $n = 8$) and point mutation of the cyclin D1 Tcf site at −81 abolished induction by both β-catenin Y33 and by p110α-CAAX (Figure 2, B and C). The twofold induction of −1745CD1LUC by β-catenin Y33 in DU145 is consistent with the threefold induction of cyclin D1 promoter activity described in Hela cells (Tetsu and McCormick, 1999).

To investigate the signaling pathways by which PI3K induced cyclin D1, we used previously well-characterized dominant negative mutant expression vectors. In agreement with a previous study in which expression of Rac1-N17 blocked PI3K-induced activity (Rodriguez-Viciana *et al.*, 1997), p110-CAAX–induced cyclin D1 promoter activity was reduced 50% by Rac1-N17 (Figure 2D). Because PI3K activates Akt (Franke *et al.*, 1995; King *et al.*, 1997), we examined the role of Akt in PI3K induction of cyclin D1. A kinase-inactive dominant negative Akt (Akt K179 M), but not wild-type Akt, inhibited p110α-CAAX–induced activation of cyclin D1 (Figure 2D). Because Akt regulates several distinct pathways including NF-κB activity (Kane *et al.*, 1999; Romashkova and Makarov, 1999), we examined the possibility that IKK activity may play a role in PI3K induction of cyclin D1. The dominant IκB inhibitor, CMV-IκBαSr, inhibited p110α-CAAX–induced activation of cyclin D1 (Figure 2D) but did not inhibit c-*fos* LUC activity (our unpublished results). The p38 MAPK inhibitor SB203580, the ERK inhibitor PD98059, and rapamycin had no effect on p110α-induced D1 activity (our unpublished results). Tcfs may serve as either activators or repressors of gene transcription through the Tcf site (Bienz, 1998; Barker *et al.*, 2000). In DU145 cells we found that mutation of the cyclin D1 Tcf site reduced the basal promoter activity to 55%, consistent with previous studies suggesting the cyclin D1 Tcf site functions as a basal enhancer element in several cell types (Shtutman *et al.*, 1999; D'Amico *et al.*, 2000; Soriano *et al.*, 2001; Figure 2E). Coexpression of a DN-Tcf, but not wild-type Tcf, inhibited p110-CAAX induced cyclin D1 promoter activity (Figure 2F). Together these studies suggest p110-CAAX induction of cyclin D1 promoter activity involves a Tcf signaling pathway.

Cyclin D1 Is Required for PI3K-dependent S-Phase Entry in Primary Cells

The current studies suggest cyclin D1 is a distal target of PI3K in serum-induced DNA synthesis. Cyclin D1 is known to play a role in the entry of cells into the DNA synthetic (S) phase induced by several growth factors and mitogens. The role of PI3K in serum-induced DNA synthesis through cyclin D1 is not known and was therefore further examined. In wt MEFs, serum-induced entry into S phase, increased from 10 to 26% (Figure 3A). LY294002 reduced the S-phase proportion from 26 to 7% at 24 h, indicating that serum-induced DNA synthesis is substantially PI3K dependent in MEFs (Figure 3, A and B). LY294002 treatment reduced serum-induced DNA synthesis by a mean of 39% at 12 h after serum addition but did not affect the serum-induced entry into the S-phase fraction in the *cyclin D1*$^{-/-}$ MEFs (Figure 3C, mean for $n = 4$ separate experiments). To confirm that LY294002 was effective at inhibiting signaling downstream of PI3K in both the *cyclin D1* wt and *cyclin D1*$^{-/-}$ MEFs, western blotting was performed for phosphorylated Akt using a specific antibody, and the membrane was probed for total ERK as a control (Figure 3D). Serum-induced phosphorylation of Akt was reduced by LY294002 in both cell types (Figure 3D). Similar analyses of serum-induced DNA synthesis were performed in 3T3 cells derived from the *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ MEFs with similar results (our unpublished results). To determine the role of PI3K in apoptosis mediated by serum deprivation, annexin V staining and sub G_1 analysis was performed on the MEFs. *Cyclin D1*$^{-/-}$ MEFs exhibited a fivefold greater level of annexin V staining compared with wt MEFs, indicating increased basal apoptosis as previously shown (Albanese *et al.*, 1999) that was rescued by serum (Figure 3E). LY294002 did not affect the level of apoptosis in either wt or *cyclin D1*$^{-/-}$ MEFs as

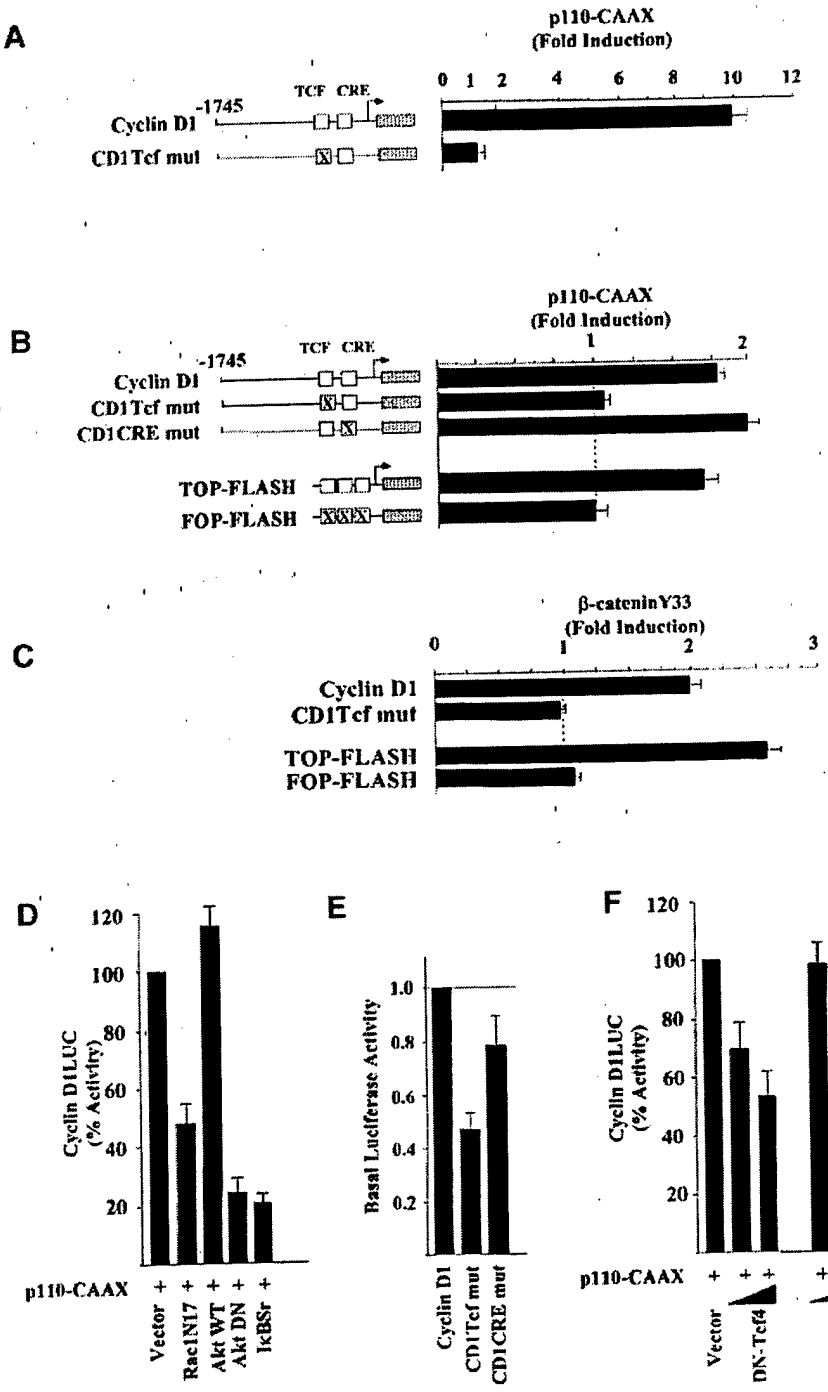


Figure 2. PI3K-induction of cyclin D1 requires the Tcf site and is dependent upon IκB. (A) The effects of the p110α-CAAX expression plasmid on the activity of the cyclin D1 promoter (−1745 CD1LUC) or of a point mutant of the Tcf site at −81 (−1745 Tcf mut) in DU145 cells grown to either > 90% or (B) 30% confluence. Regulation of the cyclin D1 and Tcf-responsive (TOPFLASH) and mutant (FOPFLASH) reporter constructs by either the p110-CAAX plasmid or (C) the activated β-catenin point mutant (Y33). The fold induction of the luciferase reporter activity is shown for at least nine separate experiments as mean ± SEM throughout. (D) The cyclin D1 promoter activity in the presence of p110α-CAAX is shown as 100% and is compared with the effect of cotransfected dominant negative inhibitors of PI3K including RacN17, Akt wt, AktDN (K179 M), or IκBαSr. The results are shown compared with equal amounts of empty control vector for each expression vector plasmid. (E) Point mutations of the cyclin D1 promoter Tcf or CRE site were compared with the basal promoter activity of −1745 CD1LUC. The activity of the wild-type promoter construction was set as 1.0. The data are mean ± SEM of five separate transfections. (F) The cyclin D1 promoter activity in the presence of p110α-CAAX (100%) is compared with the effect of dominant negative or wild-type Tcf.

determined by either annexin V staining (Figure 3E) or sub G_1 analysis (our unpublished results). These studies suggest that a substantial component of serum-induced expression of cyclin D1 is PI3K dependent and that MEFs derived from animals deleted of the *cyclin D1* gene show reduced PI3K-dependent induction of DNA synthesis.

IKKα, but not IKKβ Induces Cyclin D1 through β-Catenin/Tcf

The studies described above indicate that the PI3K activation of cyclin D1 involves Akt and IκB (Ozes *et al.*, 1999). As IKKs regulate IκB activity, we assessed the role of IKKs in

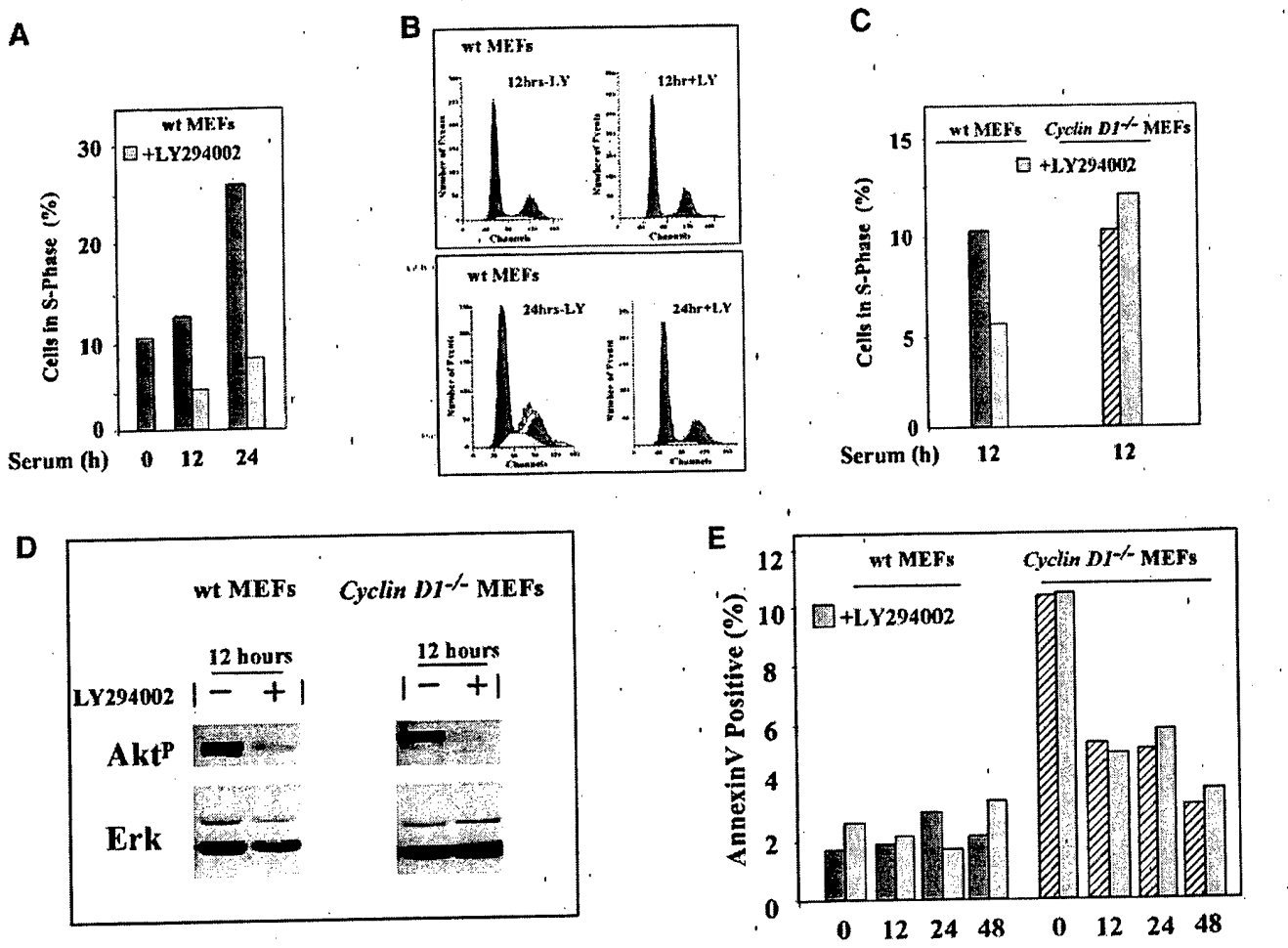


Figure 3. Involvement of cyclin D1 in PI3K-dependent S-phase entry in primary cells. (A) MEFs were treated either with vehicle (DMSO) or LY294002 (20 μM) and DNA synthesis assessed by FACS. (B) FACS analysis of wt MEFs in the presence and absence of LY294002 (LY; 20 μM) 12 and 24 h after serum stimulation. (C) The effects of LY294002 on S-phase wt and *cyclin D1*$^{-/-}$ MEFs are shown after serum stimulation as mean of four,separate experiments. (D) Western blotting for phosphorylated Akt or total ERK of MEFs treated with serum and either vehicle or LY294002. (E) The level of apoptosis was determined by Annexin V staining in serum-starved–stimulated *cyclin D1*wt and *cyclin D1*$^{-/-}$ MEFs.

PI3K-dependent activation of cyclin D1 using previously characterized dominant negative IKK mutants (Delhase *et al.*, 1999). These expression vectors behaved as previously described in cultured cells (below). We found that both the dominant negative and kinase dead IKKα constructs reduced PI3K-induced cyclin D1 promoter activity and the basal promoter activity in a dose-dependent manner (Figure 4A). The constitutively active mutant IKKαCA(S176/180E) induced the cyclin D1 promoter 4.2-fold (Figure 4B). The IKKαCA expression vector was previously well characterized and was shown to integrate in the IKK kinase using the identical transfection approach (DiDonato *et al.*, 1997; Zandi *et al.*, 1997). In contrast with IKKα, the constitutively active IKKβ mutant (IKKβCA) decreased the cyclin D1 promoter activity (see below). Using a series of 5′ promoter deletion constructions the IKKα responsiveness was confined to the proximal −163 base pairs (our unpublished results). Mutation of the Tcf site in the context of the −1745-base pair fragment abolished induction of cyclin D1 by IKKαCA (Figure 4C). IKKαCA induced TOP-FLASH threefold but did not induce a reporter with mutations of the Tcf site (FOP-LUC; Figure 4C). IKKαCA also activated the canonical NF-κB–responsive sequences (3xRelLUC) to the same extent (Figure 4C). Consistent with previous studies, in which PI3K and Akt induced NFκB activity in response to IL-1 (Madrid *et al.*, 2000, 2001), the IKKα kinase dead and dominant negative mutants reduced the activity of the NFκB-responsive reporter gene 3XRelLUC in the presence of p110α-CAAX (Figure 4D).

To provide genetic evidence for the involvement of IKKα activity in regulating cyclin D1, MEFs from *IKKα*$^{-/-}$ mice were selected by the 3T3 protocol. Cells were serum starved for 24 h and western blotting was performed to determine cyclin D1 levels. Immunostaining for IKKα showed the pres-

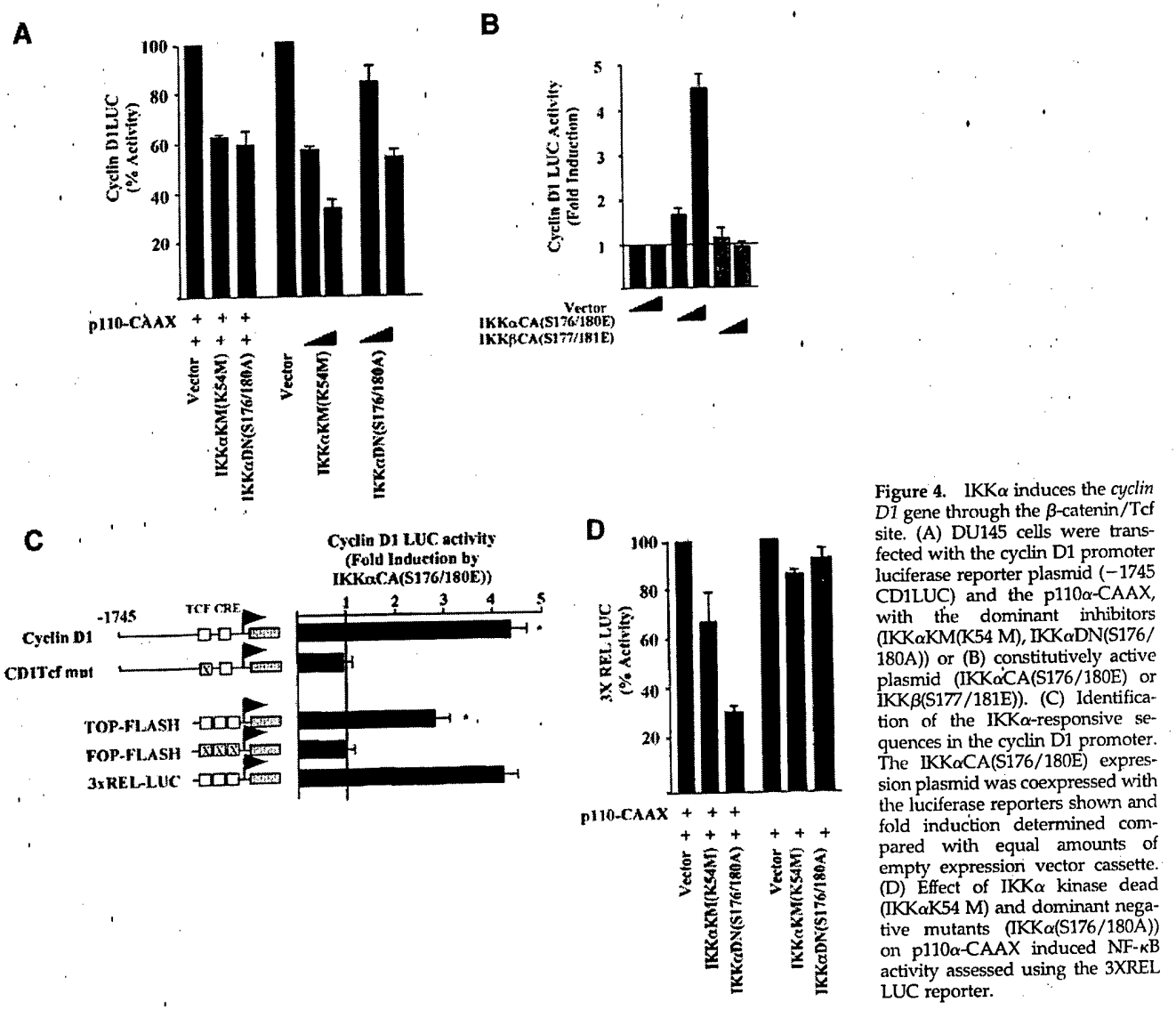


Figure 4. IKKα induces the *cyclin D1* gene through the β-catenin/Tcf site. (A) DU145 cells were transfected with the cyclin D1 promoter luciferase reporter plasmid (−1745 CD1LUC) and the p110α-CAAX, with the dominant inhibitors (IKKαKM(K54 M), IKKαDN(S176/180A)) or (B) constitutively active plasmid (IKKαCA(S176/180E) or IKKβ(S177/181E)). (C) Identification of the IKKα-responsive sequences in the cyclin D1 promoter. The IKKαCA(S176/180E) expression plasmid was coexpressed with the luciferase reporters shown and fold induction determined compared with equal amounts of empty expression vector cassette. (D) Effect of IKKα kinase dead (IKKαK54 M) and dominant negative mutants (IKKα(S176/180A)) on p110α-CAAX induced NF-κB activity assessed using the 3XREL LUC reporter.

ence of IKKα in the wt 3T3 and the absence of staining in the $IKK\alpha^{-/-}$ 3T3 cells (Figure 5A). We found that cyclin D1 abundance was reduced by 85% in the $IKK\alpha^{-/-}$ cells (Figure 5A), and the activity of the cyclin D1 promoter in the $IKK\alpha^{-/-}$ cells was lower by 67% compared with $IKK\alpha^{+/+}$ cells (Figure 5B). Serum treatment induced cyclin D1 abundance in wt MEFs by two- to threefold after 3 h, whereas in the $IKK\alpha^{-/-}$ cells, induction was delayed until 6 h after serum stimulation (Figure 5C), suggesting a role for IKKα in both the basal level of cyclin D1 expression and in serum-induced cyclin D1 abundance. Because IKKα induced cyclin D1 through the Tcf site and serum-induction of cyclin D1 protein abundance was defective in the $IKK\alpha^{-/-}$ cells, we assessed the role of the cyclin D1 promoter Tcf site in serum-induced activity. In wt 3T3 cells, serum-induced activation of the cyclin D1 promoter was reduced more than 90% by mutation of the Tcf site (Figure 5D). Furthermore, serum-induced activation of the cyclin D1 promoter was defective in the $IKK\alpha^{-/-}$ cells (Figure 5D).

Consistent with the reduced abundance of cyclin D1 in the $IKK\alpha^{-/-}$ 3T3 cells and the ability of cyclin D1 overexpression to promote DNA synthesis in fibroblasts (Pagano *et al.*, 1994), serum-induced DNA synthesis was reduced in $IKK\alpha^{-/-}$ 3T3 cells (Figure 6A). To determine whether the reduction in IKKα abundance was important in the reduced levels of cyclin D1, the $IKK\alpha^{-/-}$ 3T3 cells were transfected with the IKKαCA expression vector and MACS-sorted, and the cell extracts were subjected to Western blotting. IKKα protein levels were increased in the IKKαCA-transfected $IKK\alpha^{-/-}$ 3T3 cells (Figure 6B, lane 3). Although the relative levels of IKKα in the $IKK\alpha^{-/-}$ 3T3 cells transfected with the IKKαCA expression vector were substantially less than the wt 3T3 cells, cyclin D1 levels were increased threefold compared with

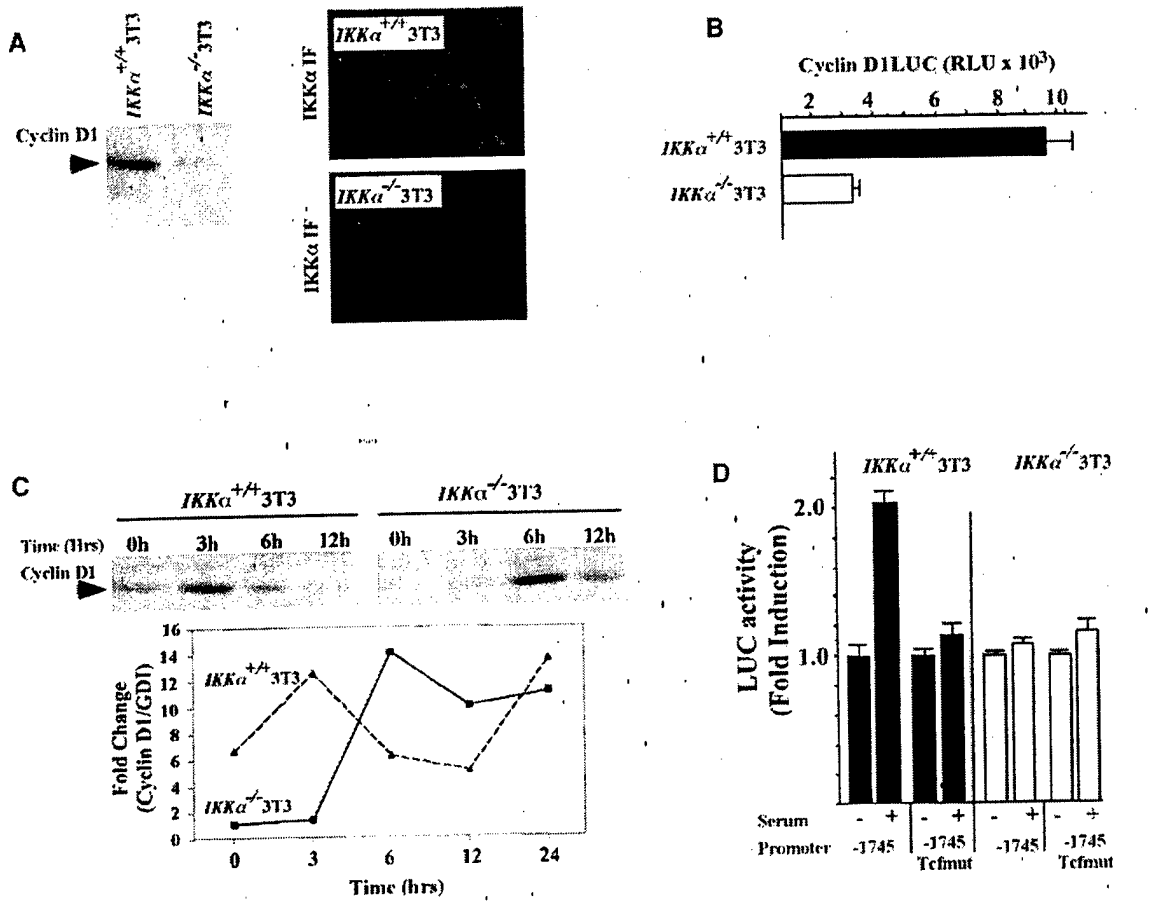


Figure 5. Reduced mitogen-induced cyclin D1 expression in $IKK\alpha^{-/-}$ cells involves Tcf signaling. (A) 3T3 cells from either wt or $IKK\alpha^{-/-}$ mice were examined by Western blotting for cyclin D1, using equal amounts of total protein. Immunostaining for IKKα in the wt or $IKK\alpha^{-/-}$ 3T3 cells. (B) The cyclin D1 promoter luciferase reporter plasmid (−1745 CD1LUC) was transfected into either wt or $IKK\alpha^{-/-}$ 3T3 cells along with the β-galactosidase control reporter. Relative cyclin D1 promoter activity is shown as mean ± SEM for n = 3. (C) Western blotting for cyclin D1 of wt or $IKK\alpha^{-/-}$ 3T3 cells treated with serum for the time points indicated. The fold change in cyclin D1 protein levels is shown normalized to GDI as a loading control. The data is representative of three separate experiments. (D) The cyclin D1 promoter (−1745CD1LUC) or the point mutant of the Tcf site (−1745Tcfmut) were compared for relative activity in wt or $IKK\alpha^{-/-}$3T3 cells. The data are mean ± SEM, n = 9.

the $IKK\alpha^{-/-}$ 3T3 cells, demonstrating a key role for IKKα in inducing cyclin D1 levels. Activity of the cyclin D1 promoter was also increased threefold in $IKK\alpha^{-/-}$ 3T3 cells transfected with the IKKαCA expression vector. Furthermore, the induction of cyclin D1 by IKKαCA required the Tcf site (Figure 6C).

To determine whether the activity of other known β-catenin responsive promoters were regulated by IKKα, the relative activity of the c-*Myc* (He *et al.*, 1998) and *Engrailed* (McGrew *et al.*, 1999) promoters were compared in the wt and $IKK\alpha^{-/-}$ 3T3 cells, with relative activity normalized to an internal control of renilla luciferase activity. The relative activity of the *Engr* and c-*Myc* promoter activity was reduced 10- to 12-fold in the $IKK\alpha^{-/-}$ 3T3 cells (Figure 6D). Furthermore, as with the cyclin D1 promoter, the serum-induced activity of the *Engr* promoter was substantially reduced in the IKKα3T3 cells (Figure 6D). Together, these studies demonstrate that the activity of both heterologous and natural Tcf responsive genes is dependent on IKKα in vivo. Furthermore, these studies demonstrate an important role for IKKα in regulating the kinetics of serum-induced expression of β-catenin/Tcf-responsive genes.

IKKα Associates with and Phosphorylates β-Catenin and Increases β-Catenin Abundance

In addition to the differences in Tcf-mediated activation of gene promoters, several lines of evidence suggest that IKKα and IKKβ fulfill distinct cellular functions. Thus, homozygous deletion of the IKKα and IKKβ genes results in distinct phenotypes (Hu *et al.*, 1999, 2001; Li *et al.*, 1999a; Takeda *et al.*, 1999), and IKKβ acts more potently on IκB proteins and plays a more significant role in the NF-κB pathway in response to activation with TNF-α and IL-1 than IKKα (Delhase *et al.*, 1999; Li *et al.*, 1999a, 1999b). To investigate further

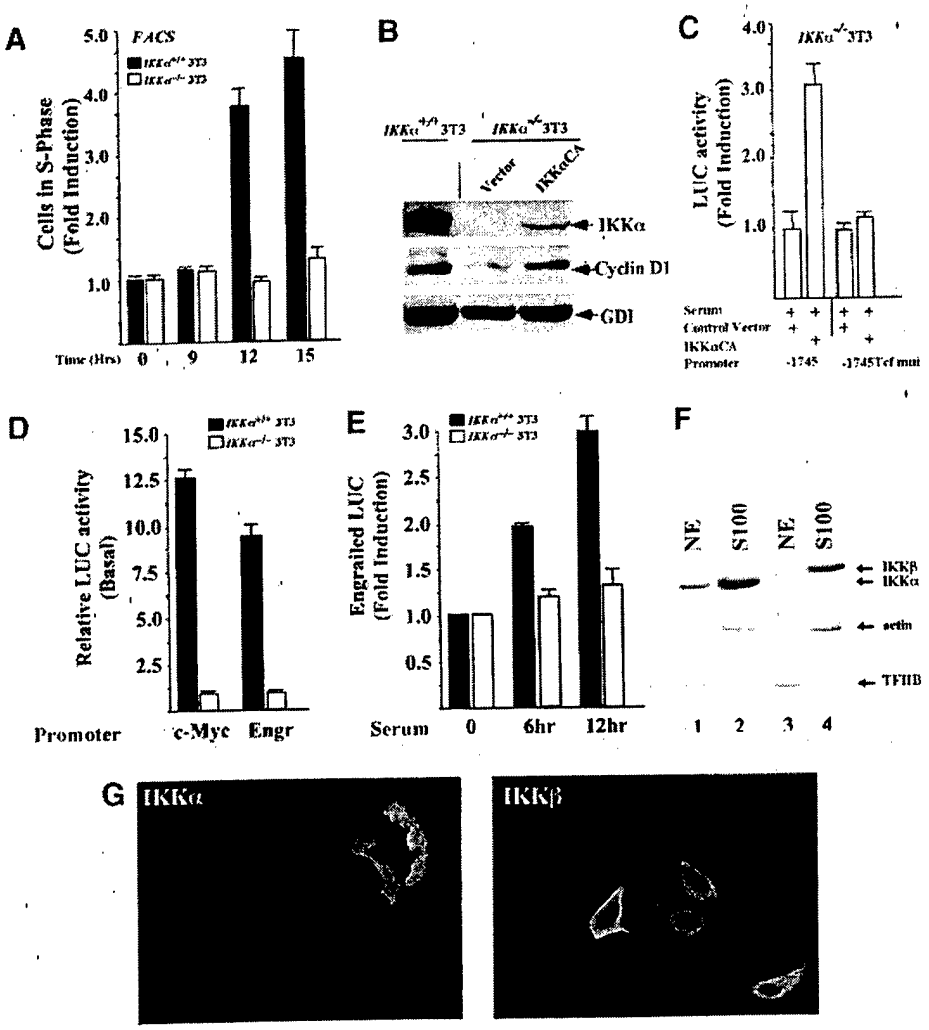


Figure 6. IKKα regulates mitogen-induced DNA synthesis and is required for Tcf signaling to natural Tcf-responsive genes. (A) The serum-induced S-phase fraction of wt or *IKKα*$^{-/-}$ 3T3 cells was compared. The data are mean ± SEM, N = 7. (B) Western blotting of wt or *IKKα*$^{-/-}$ 3T3 cells transfected with an expression vector for IKKαCA or empty expression vector. IKKα and cyclin D1 immunoblotting is shown with GDI as an internal control for loading. (C) Relative activity of the cyclin D1 promoter or the corresponding Tcf point mutant in *IKKα*$^{-/-}$ 3T3 cells transfected with either IKKα expression vector or the control vector. The data are mean ± SEM, n = 8. (D) Relative activity of the *c-Myc* and *Engr* promoters (n = 6) in randomly cycling wt or *IKKα*$^{-/-}$ 3T3 cells. The activity of the promoter is set as 1 in the *IKKα*$^{-/-}$ 3T3 cells. (E) Activity of the *Engr* promoter in the presence of serum stimulation. The data is mean ± SEM of n = 12 separate experiments. (F) Nuclear and cytosolic fractions of Cos-7 cells were analyzed by Western blotting for the abundance of IKKβ or IKKα in the nuclear (NE) and cytosolic (S100) fractions. Internal controls for (nuclear; TFIIB) and cytoplasmic (actin) markers are shown. Substantially more IKKα than IKKβ was found in the nuclear extracts (NE) of Cos-7 cells. (G) Immunostaining for IKKα and IKKβ. IKKβ is predominantly extranuclear, whereas IKKα was found in both nuclear and cytoplasmic compartments.

the basis for these diverse functions, we determined the subcellular localization of IKKα and IKKβ and their cell-type expression patterns. Western blot analysis of nuclear and cytoplasmic extracts showed a differential localization of IKKα and IKKβ in Cos-7 cells with IKKα present in both the nuclear and cytoplasmic fractions (marked by TFIIB and actin, respectively), whereas IKKβ was predominantly cytoplasmic (Figure 6A), consistent with the immunohistochemical analysis (Figure 6B).

Consistent with a role for IKKα in regulating β-catenin phosphorylation and/or abundance, the total level of wt β-catenin and of a higher molecular weight form of β-catenin were increased in cells coexpressing IKKαCA and β-catenin expression vectors (Figure 7A). Point mutation of β-catenin at Ser33 to alanine abrogated the induction of the higher molecular weight form of β-catenin (Yost *et al.*, 1996). The abundance of the β-catenin S37A mutant and the higher molecular weight form were also increased in cells transfected with IKKαCA, suggesting a dominant role for S33 in the generation of the high molecular weight form. Because IKKαCA induced cyclin D1 and Tcf reporter activity, we hypothesized that IKKα may regulate β-catenin abundance and/or phosphorylation. In our previous studies, IKK immunoprecipitation on fractionated Cos-7 cell extracts cotransfected with HA-tagged β-catenin and FLAG-tagged IKKα showed that β-catenin is present in IKKα immunoprecipitates and IKKα was also present in β-catenin immunoprecipitates (Lamberti *et al.*, 2001). We had also demonstrated an association between endogenous β-catenin and IKKα by reciprocal IP-Western blotting of SW480 cell extracts (Lamberti *et al.*, 2001). Consistent with these findings in cultured cells, we found that GST-β–catenin fusion proteins were efficient substrates for phosphorylation by IKKα in vitro in which IKKα was immunoprecipitated from cultured cells and used as the enzyme source (our unpublished results). The minimal region of β-catenin sufficient for phosphorylation by immunoprecipitated IKKα included the N-terminal portion of the molecule between aa 30 and 55 (our unpublished results). IKKα bound to and phosphorylated β-catenin in vitro with an efficiency that was similar to that of IκB as recently shown (Lamberti *et al.*, 2001).

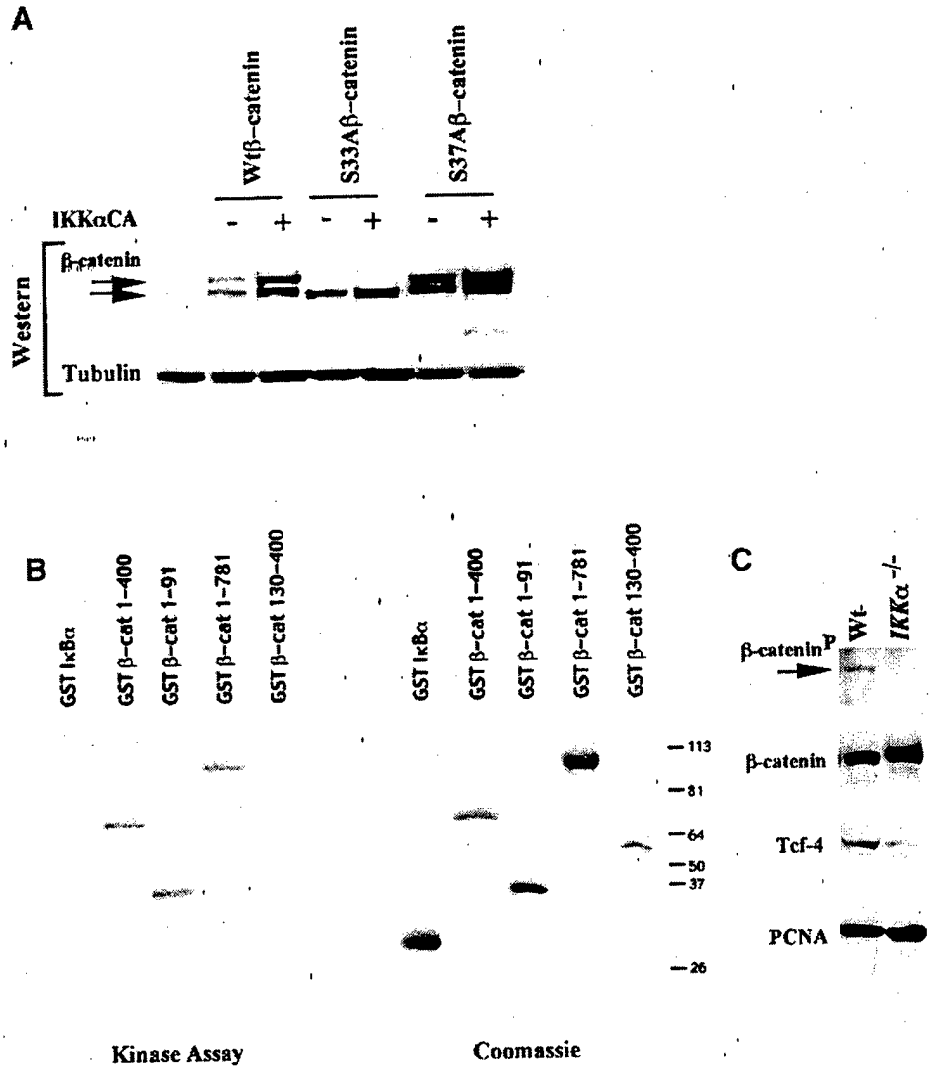


Figure 7. IKKα phosphorylates β-catenin and increases β-catenin abundance. (A) The IKKαCA(S/E) expression plasmid was coexpressed in cells transfected with either wild-type or mutants (S33A, S37A) of β-catenin. Western blotting analysis showed an increase the total amount of β-catenin, including a higher molecular weight form (upper arrow). The S33Aβ-catenin shows no increase in the amount of the high molecular weight form. (B) IKKα kinase assays conducted using baculovirus expressed purified IKKα and the GST-β-catenin constructs as shown. Kinase activity (left panel) and the Coomassie stained gel for the substrate are shown. (C) Western blot analysis of *IKKα*$^{-/-}$ or *IKK*wt 3T3 cells with antibodies to phosphospecific β-catenin, total β-catenin, Tcf-4, and PCNA.

Because the IKKα immunoprecipitation may coprecipitate other components of the IKK complex to phosphorylate β-catenin, IKKα was produced in baculovirus, purified, and used as the enzyme source in IKKα kinase assays with β-catenin as substrate (Figure 7B). Purified IKKα was sufficient for phosphorylation of GST-β-catenin 1–400. Deletion of the N-terminus of β-catenin (130–400) abolished phosphorylation by IKKα, and the N-terminus from 1–91 was sufficient for phosphorylation by IKKα (Figure 7B). To determine if the endogenous IKKα is involved in the phosphorylation of β-catenin, equal amounts of proteins from *IKKα*$^{-/-}$ and wt MEFs were compared using an antiphospho-β-catenin antibody. The results shown in Figure 7C demonstrated that phosphorylated β-catenin exists in wt MEFs but with a significantly reduced abundance in the *IKKα*$^{-/-}$ cells. Interestingly, the levels of the nuclear effector of β-catenin, Tcf were also lower in the *IKKα*$^{-/-}$ cells. The abundance of the nuclear protein PCNA was similar between the *IKKα*$^{-/-}$ and wt MEFs.

Our findings that IKKα phosphorylates β-catenin and that IKKαCA increases Tcf activity and β-catenin abundance suggests that β-catenin phosphorylation by IKKα may contribute to the regulation of β-catenin–mediated Tcf-dependent gene transcription. The consequent induction of cyclin D1 by PI3K-IKKα-Tcf signaling contributes to the induction of DNA synthesis.

DISCUSSION

This study demonstrates for the first time a requirement for IKKα in response to mitogens and DNA synthesis and the induction thereby of cyclin D1 abundance and promoter activity through a β-catenin/Tcf pathway. IKKα selectively and directly induced cyclin D1 but not cyclin E or cyclin A. Reintroduction of IKKα into IKKα-deficient cells restored cyclin D1 expression and promoter activity in a Tcf-dependent manner. Using a dominant negative mutant of Tcf

activity we showed that IKKα induction of cyclin D1 requires β-catenin/Tcf activity. IKKα was shown to be a key genetic determinant of the activity of several other Tcf responsive genes (*c-Myc, Engr, TcfLUC*). IKKα-deficient cells demonstrated a delayed induction of serum-induced DNA synthesis and a delayed induction of serum-induced activity of the *cyclin D1* and *Engr* promoters. Together these studies indicate a key role for *IKKα* in coordinating the kinetics of mitogen responsiveness to a subset of cellular targets. These studies are consistent with an evolving view that separate components of the IKK complex may subserve distinct functions to convey signal transduction specificity (Ghosh and Karin, 2002).

Serum induction of DNA synthesis and cyclin D1 expression was PI3K dependent, and cyclin D1 was required for the PI3K-dependent induction of DNA synthesis. PI3K-dependent, serum-induced DNA synthesis was substantially reduced in *cyclin D1*-deficient cells, indicating a key role for cyclin D1 in this signaling pathway. Although serum deprivation increased apoptosis in *cyclin D1*$^{-/-}$ MEFs, the inhibition of apoptosis by serum addition was not affected by PI3K inhibition, demonstrating distinct functions of cyclin D1 in PI3K-dependent proliferation versus apoptosis. Although the upstream effectors of IKKα that contribute to the induction of β-catenin remain to be identified, the current studies demonstrate that the PI3K-dependent induction of cyclin D1 involves IKKα. PI3K is involved in a PDGF-regulated pathway that activates Akt, leading to an association with and activation of IKKα in cultured cells (Romashkova and Makarov, 1999), which is consistent with a role for PI3K in activating a subset of IKKα functions. Although IκB-independent effects of Akt on NF-κB have been reported (Madrid *et al.*, 2000; Reddy *et al.*, 2000) and IKKα phosphorylation by Akt is not essential for IKK activation of NF-κB signaling (Delhase and Karin, 2000), increasing evidence suggests IKKα conveys important kinase-dependent and -independent functions. Because the dominant inhibitors of Akt, IKKα, and Tcf reduced the induction of cyclin D1 by constitutively active PI3K mutants, it appears that PI3K may be an important upstream inducer of IKKα in the context of β-catenin/Tcf signaling.

The current studies identify the cyclin D1 Tcf site as the common target of activated PI3K, IKKα, and β-catenin and establish, using dominant negative mutants, a colinearity of these components to regulate cyclin D1 expression in cultured cells. Wnt family ligands and Frizzled family receptors define one important mechanism that can induce β-catenin/Tcf signaling (Polakis, 2000a). Although until quite recently, the activity of the β-catenin/Tcf pathway was thought to be regulated only by Wnts, a substantial body of evidence suggests that important additional regulators of this pathway exist. The protein encoded by *Gas6*, a growth factor of the vitamin K–dependent family, which binds the Axl receptor of the tyrosine kinase family, stabilizes β-catenin and induces Tcf signaling (Goruppi *et al.*, 2001). Hepatocyte growth factor/scatter factor (Papkoff and Aikawa, 1998) and oncogenic mutations of RON and MET (Danilkovitch-Miagkova *et al.*, 2001) all increase cytosolic β-catenin and activate Lef/Tcf-responsive reporters. The *Xenopus* wnt target gene *twin* is induced by SMAD4 through the β-catenin/Tcf complex (Nishita *et al.*, 2000). In addition, suppressor screens in *Drosophila* have identified *Dpresenilin* as a target of *Armadillo* (a homolog of β-catenin; Cox *et al.*, 2000) and a cell-adhesion–dependent pathway involving the integrin-linked kinase (ILK) was also shown to regulate β-catenin levels and activity (Lin and Perrimon, 1999; Payre *et al.*, 1999; Tsuda *et al.*, 1999). The *cyclin D1* gene, which plays a critical role in oncogenic signaling pathways, is regulated (via its Tcf site) by several components that can also regulate β-catenin/Tcf signaling. Previous studies have demonstrated a role for the β-catenin-Tcf signaling pathway in activation of the *cyclin D1* gene by expressing mutants of β-catenin, Wnt-1, ILK and repression via presenilin 1 (PS1) or HBP1 (Shtutman *et al.*, 1999; Barker *et al.*, 2000; Lin *et al.*, 2000; Soriano *et al.*, 2001). Although the *cyclin D1* gene is known to be regulated by Tcf signaling, the upstream activators of this pathway were not known. Through identifying the β-catenin/Tcf site of the cyclin D1 promoter as a target of PI3K and IKKα signaling the current studies provide important evidence for a new signaling pathway that regulates β-catenin signaling and cyclin D1 expression.

The reduction of cyclin D1 promoter activity and protein abundance in the *IKKα*$^{-/-}$ cells in the current studies, provides genetic evidence for IKKα as an inducer of cyclin D1 abundance. Recent studies also provided genetic evidence that this IKKα dominant negative mutant inhibits cyclin D1 expression (Cao *et al.*, 2001). *IKKα*^{AA} knockin mice, in which the IKKα catalytic subunit activation loop serines were substituted for alanines, exhibited reduced cyclin D1 abundance in the mammary gland (Cao *et al.*, 2001). The *IKKα*^{AA} knockin mice failed to develop normal lobular alveolar architecture during pregnancy, and the mammary gland phenotype resembled that of the *cyclin D1*$^{-/-}$ mice (Cao *et al.*, 2001), providing further support for a genetic link between IKKα and cyclin D1. The current studies are important in identifying *cyclin D1* as a direct, rather than an indirect, transcriptional target of IKKα and extend these observations by identifying the molecular mechanisms by which IKKα directly induces cyclin D1.

Several lines of evidence in the current studies demonstrate a key role for IKKα in activating β-catenin/Tcf signaling at the cyclin D1 promoter. First, the reduced nuclear Tcf abundance in the *IKKα*$^{-/-}$ cells provides genetic evidence supporting an important role for IKKα in activating β-catenin/Tcf signaling. Second, activating mutants of IKKα induced the cyclin D1 promoter significantly (4.7-fold) and induced Tcf activity assessed as an heterologous reporter linked to the luciferase reporter gene. Third, mutation of a single Tcf site at −81 abrogated induction of the cyclin D1 promoter by either IKKα or an activating mutation of β-catenin. Together, these studies demonstrate that IKKα induces Tcf signaling through this site. In recent studies, IKK immunopreciptiated from cultured cells phosphorylated β-catenin as a substrate in vitro (Lamberti *et al.*, 2001). As IKK immunoprecipitation coprecipitates IKKα, IKKβ, and IKKγ (NEMO), these findings raised the important question of whether IKKα alone phosphorylated IKKα.

In the current studies both transfected and endogenous IKKα was found in association with β-catenin in a variety of cultured cells. A constitutively active form of IKKα increased both β-catenin abundance and phosphorylation and induced Tcf-dependent transcription. Phosphorylation of β-catenin by IKKα required the N-terminus of β-catenin, and its phosphorylation apparently contributed to the in-

crease in β-catenin levels. This effect of IKKα on β-catenin was dependent, at least in part, on the S33 residue of β-catenin that has an important role in regulating β-catenin stability. The N-terminal domain of β-catenin containing these serine residues was sufficient for in vitro phosphorylation and is identical to the domain found in the N-terminus of IκBα that is phosphorylated by the IKK complex (Aberle *et al.*, 1997, Orford *et al.* 1997). Phosphorylation of serine residues 33 and 37 of β-catenin has been implicated in regulating protein stability and signaling by GSK3β (Yost *et al.*, 1996). Mutation of β-catenin at S33 abrogated the ability of IKKα to induce the higher molecular weight form of β-catenin and partially reduced the increase in β-catenin levels. Because the IKKα-dependent phosphorylation of β-catenin activated Tcf signaling, this effect most probably represents a different means by which β-catenin is regulated either by GSK3β-β-TrCP or Siah-1 (Polakis, 2000a).

Cyclin D1 and β-catenin overexpression correlates with poor prognosis in human breast cancer, suggesting a role for cyclin D1 in β-catenin/Tcf-mediated signaling and cell transformation (Shtutman *et al.*, 1999; Barker *et al.*, 2000; Lin *et al.*, 2000). Aberrant activation of the Tcf pathway by mutations in components of the Wnt-signaling pathway is believed to contribute to the development of a variety of human cancers, including colon, breast, and prostate cancer (Polakis, 2000b). IKKα appears to provide an important link to the control of *cyclin D1* gene expression through induction of Tcf signaling. Understanding the precise mechanism by which IKKα regulates β-catenin signaling and the factors specifying IKK activity on β-catenin compared with NF-κB remains pivotal in determining the signal transduction specificity regulated by these two important pathways.

ACKNOWLEDGMENTS

We thank Drs K. Akama, D. Ballard, M. Karin, F. McCormick, F. Mercurio, and B. Vogelstein for plasmids and cells. This work was supported by grants to R.G.P. from National Institutes of Health (RO1CA70896, RO1CA75503, RO1CA86072, RO1CA86071), the Breast Cancer Alliance Inc., and The Susan Komen Breast Cancer Foundation. R.G.P. is a recipient of the Weil Caulier Irma T. Hirschl Career Scientist award and was the Diane Belfer Faculty Scholar in Cancer Research. C.A. is a recipient of RO3 AG20337. S.B. is supported by the Susan G. Komen Breast Cancer Foundation and the Department of Defense.

REFERENCES

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-Catenin is a target for the ubiquitin-proteasome pathway. EMBO J. *16*, 3797–3804.

Albanese, C. *et al.* (1999). Activation of the *cyclin D1* gene by the E1A-associated protein p300 through AP-1 inhibits cellular apoptosis. J. Biol. Chem. *274*, 34186–34195.

Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R.G. (1995). Transforming $p21^{ras}$ mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J. Biol. Chem. *270*, 23589–23597.

Amanatullah, D., Zafonte, B., Albanese, C., Fu, M.-F., Messiers, C., Hassell, J., and Pestell, R.G. (2001). Ras regulation of cyclin D1 promoter. Methods Enzymol. *332*, 116–127.

Ashton, A.W., Watanabe, G., Albanese, C., Harrington, E.O., Ware, J.A., and Pestell, R.G. (1999). Protein kinase Cδ inhibition of S-phase transition in capillary endothelial cells involves the cyclin dependant kinase inhibitor $p27^{Kip1}$. J. Biol. Chem. *274*, 20805–20811.

Barker, N., Morin, P.J., and Clevers, H. (2000). The yin-yang of TCF/β-catenin signaling. Adv. Cancer Res. *77*, 1–4.

Bienz, M. (1998). TCF: transcriptional activator or repressor. Curr. Opin. Cell. Biol. *10*, 366–372.

Brockman, J.A., Scherer, D.C., McKinsey, T.A., Hall, S.M., Qi, X., Lee, W.Y., and Ballard, D.W. (1995). Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. Mol. Cell. Biol. *15*, 2809–2818.

Bromberg, J.F., Wrzeszczynska, M.H., Devgan, G., Zhao, Y., Pestell, R.G., Albanese, C., and Darnell, J.E. (1999). Stat3 as an oncogene. Cell *98*, 295–303.

Brown, J.R., Nigh, E., Lee, R.J., Ye, H., Thompson, M.A., Saudou, F., Pestell, R.G., and Greenberg, M.E. (1998). Fos family members induce cell cycle entry by activating cyclin D1. Mol. Cell. Biol. *18*, 5609–5619.

Cadigan, K.M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes Dev. *11*, 3286–3305.

Cao, Y., Bonizzi, G., Seagroves, T., Greten, F.R., Johnson, R., Schmidt, E.V., and Karin, M. (2001). IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell *107*, 763–775.

Chang, H.W., Aoki, M., Fruman, D., Auger, K.R., Bellacosa, A., Tsichlis, P.N., Cantley, L.C., Roberts, T.M., and Vogt, P.K. (1997). Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. Science *276*, 1848–1850.

Cox, R.T., McEwen, D.G., Myster, D.L., Duronio, R.J., Loureiro, J., and Peifer, M. (2000). A screen for mutations that suppress the phenotype of *Drosophila armadillo*, the β-catenin homolog. Genetics *155*, 1725–1740.

D'Amico, M. *et al.* (2000). The integrin-linked kinase regulates the cyclin D1 gene through glycogen synthase kinase 3β and CREB-dependent pathways. J. Biol. Chem. *275*, 32649–32657.

Danilkovitch-Miagkova, A., Miagkov, A., Skeel, A., Nakaigawa, N., Zbar, B., and Leonard, E.J. (2001). Oncogenic mutants of Ron, and Met receptor tyrosine kinases cause activation of the β-catenin pathway. Mol. Cell. Biol. *21*, 5857–5868.

Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. Genes Dev. *13*, 2905–2927.

Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999). Positive and negative regulation of IκB kinase activity through IKKβ subunit phosphorylation. Science *284*, 309–313.

Delhase, M., and Karin, M. (2000). Kinase regulation in inflammatory response. Nature *406*, 367–368.

Di Cristofano, A., De Acetis, M., Koff, A., Cordon-Cardo, C., and Pandolfi, P.P. (2001). Pten and p27 KIP1 cooperate in prostate cancer tumor suppression in the mouse. Nat. Genet. *27*, 222–224.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., and Karin, M. (1997). A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature *388*, 548–554.

Fantl, V., Edwards, A.W., Steel, J.H., Vonderhaar, B.K., and Dickson, D. (1999). Impaired mammary gland development in Cyc−/− mice during pregnancy and lactation is epithelial cell autonomous. Development *212*, 1–11.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. *9*, 2364–2372.

Franke, T., Kaplan, D., Cantley, L., and Toker, A. (1997). Direct regulation of the Akt proto-oncogene product by phosphotidylinositol-3,4-bisphosphate. Science *275*, 665–668.

Franke, T.F., Yang, S.I., Chan, T.O., Datta, K., Kazlauskas, A., Morrisin, D.K., Kaplan, D.R., and Tsichlis, P.N. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell *81*, 727–736.

Fuchs, S.Y., Chen, A., Xiong, Y., Pan, Z.Q., and Ronai, Z. (1999). HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. Oncogene *18*, 2039–2046.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-κB puzzle. Cell *109*, S81–S96.

Goruppi, S., Chiaruttini, C., Ruaro, M.E., Varnum, B., and Schneide, C. (2001). Gas6 induces growth, beta-catenin stabilization, and T-cell factor transcriptional activation in contact-inhibited C57 mammary cells. Mol. Cell. Biol. *21*, 902–915.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509–1512.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.-S., Jin, O., and Woodgett, J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86–90.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science *284*, 316–320.

Hu, Y., Baud, V., Oga, T., Kim, K.I., Yoshida, K., and Karin, M. (2001). IKKα controls formation of the epidermis independently of NF-kB. Nature *410*, 710–714.

Huelsken, J., and Behrens, J. (2000). The Wnt signaling pathway. J. Cell Sci. *113*, 3545–3546.

Joyce, D., Albanese, C., Steer, J., Fu, M., Bouzahzah, B., and Pestell, R.G. (2001). NF-kB and cell-cycle regulation: the cyclin connection. Review. Cytokine Growth Factor Rev. *12*, 73–90.

Joyce, D. *et al.* (1999). Integration of Rac-dependent regulation of cyclin D1 transcription through an NF-κB-dependent pathway. J. Biol. Chem. *274*, 25245–25249.

Kane, L.P., Shapiro, V.S., Stokoe, D., and Weiss, A. (1999). Induction of NF-kB by the Akt/PKB kinase. Curr. Biol. *9*, 601–604.

Karin, M., and Delhase, M. (2000). The IκB kinase (IKK) and NF-κB: key elements of proinflammatory signaling. Semin. Immunol. *12*, 85–98.

King, W.G., Mattaliano, M.D., Chan, T.O., Tsichlis, P.N., and Brugge, J.S. (1997). Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol. Cell. Biol. *17*, 4406–4418.

Klippel, A., Escobedo, M.A., Wachowicz, M.S., Apell, G., Brown, T.W., Giedlin, M.A., Kavanaugh, W.M., and Williams, L.T. (1998). Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. Mol. Cell. Biol. *18*, 5699–5711.

Lamberti, C., Lin, K.M., Yamamoto, Y., Verma, U., Verma, I.M., Byers, S., and Gaynor, R.B. (2001). Regulation of beta-catenin function by the IkappaB kinases. J. Biol. Chem. *276*, 42276–42286.

Lee, R.J. *et al.* (2000). Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. Mol. Cell. Biol. *20*, 672–683.

Li, Q., Lu, Q., Hwang, J.Y., Buscher, D., Lee, K.F., Izpisua-Belmonte, J.C., and Verma, I.M. (1999a). IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev. *13*, 1322–1328.

Li, Z.W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999b). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J. Exp. Med. *189*, 1839–1845.

Lin, S.-Y., Xia, W., Wang, J.C., Kwong, K.Y., Spohn, B., Wen, Y., Pestell, R.G., and Hung, M.-C. (2000). β-Catenin, a novel prognostic marker for breast cancer: its role in cyclin D1 expression and cancer progression. Proc Natl. Acad. Sci. USA. *97*, 4262–4266.

Lin, X., and Perrimon, N. (1999). Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signaling. Nature *400*, 281–284.

Liu, J., Stevens, J., Rote, C.A., Yost, H.J., Hu, Y., Neufeld, K.L., White, T.L., and Matsunami, N. (2001). Siah-1 mediates a novel β-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. Mol. Cell *7*, 927–936.

Madrid, L.V., Mayo, M.W., Reuther, J.Y., and Baldwin, A.S. (2001). Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J. Biol. Chem. *276*, 18934–40.

Madrid, L.V., Wang, C.Y., Guttridge, D.C., Schottelius, A.J., Baldwin, A.S.J., and Mayo, M.W. (2000). Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kB. Mol. Cell. Biol. *20*, 1626–1638.

Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C., Downward, J., Pestell, R.G., and Kanakura, Y. (1999). Transcriptional regulation of cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. EMBO J. *18*, 1367–1377.

Matsuzawa, S.-i., and Reed J.C. (2001). Siak-1, SIP and Ebi collaborate in a novel pathway. Mol. Cell *7*, 915–926.

McGrew, L.L., Takemaru, K., Bates, R., and Moon, R.T. (1999). Direct regulation of the *Xenopus* engrailed-2 promoter by the Wnt signaling pathway, and a molecular screen for Wnt-responsive genes, confirm a role for Wnt signaling during neural patterning in *Xenopus*. Mech. Dev. *87*, 21–32.

Nishita, M., Hashimoto, M.K., Ogata, S., Laurent, M.N., Ueno, N., Shibuya, H., and Cho, K.W. (2000). Interaction between Wnt and TGF-beta signaling pathways during formation of Spemann's organizer. Nature *403*, 781–785.

Orford, K., Crockett, C., Weisman, A., and Byers, S.W. (1997). Serine phosphorylation-regulated ubiquitination and proteosomal degradation of β-catenin. J. Biol. Chem. *272*, 24735–24738.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M., and Donner, D.B. (1999). NF-kappaB activation by tumor necrosis factor requires the Akt serine-threonine kinase. Nature *401*, 82–85.

Pagano, M., Theodorus, A.M., Tam, S.W., and Draetta, G.F. (1994). Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. Genes Dev. *8*, 1627–1639.

Papkoff, J., and Aikawa, M. (1998). WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells. Biochem. Biophys. Res. Commun. *247*, 851–858.

Payre, F., Vincent, A., and Carreno, S. (1999). ovo/svb integrates Wingless and DER pathways to control epidermis differentiation. Nature *400*, 271–275.

Pestell, R.G., Albanese, C., Reutens, A.T., Segall, J.E., Lee, R.J., and Arnold, A. (1999). The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocr. Rev. *20*, 501–534.

Polakis, P. (2000a). More than one way to skin a catenin. Cell *105*, 563–565.

Polakis, P. (2000b). Wnt signaling and cancer. Genes Dev. *14*, 1837–1851.

Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D.B., Perera, S., Roberts, T.M., and Sellers, W.R. (1999). Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA *96*, 2110–2115.

Reddy, S.A., Huang, J.H., and Liao, W.S. (2000). Phosphatidylinositol 3-kinase as a mediator of TNF-induced NF-kappa B activation. J. Immunol. *164*, 1355–1363.

Rodriguez-Viciana, P., Warne, P.H., Khwaja, A., Marte, B.M., Pappin, D., Das, P., Waterfield, M.D., Ridley, A., and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by ras. Cell *89*, 457–467.

Romashkova, J.A., and Makarov, S.S. (1999). NF-κB is a target of AKT in anti-apoptotic PDGF signaling. Nature *401*, 86–90.

Sampson, E.M., Haque, Z.K., Ku, M.-C., Tevosian, S.G., Albanese, C., Pestell, R.G., Paulson, K.E., and Yee, A.S. (2001). Negative regulation of the Wnt–β-catenin pathway by the transcriptional repressor HBP1. EMBO J. *20*, 4500–4511.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA *96*, 5522–5527.

Sicinski, P. *et al.* (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell *82*, 621–630.

Soriano, S., Kang, D.E., Fu, M., Chevallier, N., Pestell, R.G., Zheng, H., and Koo, E.H. (2001). Presenilin 1 modulates β-catenin/LEF signaling independently of APP and Notch processing. J. Cell Biol. *152*, 785–794.

Takeda, K. *et al.* (1999). Limb and skin abnormalities in mice lacking IKKα. Science *284*, 313–316.

Tetsu, O., and McCormick, F. (1999). β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422–424.

Tsuda, M. *et al.* (1999). The cell-surface proteoglycan Dally regulates Wingless signaling in Drosophila. Nature *400*, 276–280.

Vanhaesebroeck, B., and Waterfield, M.D. (1999). Signaling by distinct classes of phosphoinositide 3-kinases. Exp. Cell Res. *253*, 239–254.

Watanabe, G. *et al.* (1996a). Induction of cyclin D1 by simian virus 40 small tumor antigen. Proc. Natl. Acad. Sci. USA. *93*, 12861–12866.

Watanabe, G., Lee, R.J., Albanese, C., Rainey, W.E., Batlle, D., and Pestell, R.G. (1996b). Angiotensin II (AII) activation of cyclin D1-dependent kinase activity. J. Biol. Chem. *271*, 22570–22577.

Winston, J.T., Strack, P., Beer-Romero, P., Chu, C., Elledge, S.J., and Harper, J.W. (1999). The SCF-βTRCP ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBβ and β-catenin and stimulates IκBα ubiquitination in vitro. Genes Dev. *3*, 270–283.

Yamamoto, Y., and Gaynor, R.B. (2001). Therapeutic potential of the NF-kB pathway in the treatment of inflammation and cancer. J. Clin. Invest. *107*, 135–142.

Yamamoto, Y., Yin, M.J., and Gaynor, R.B. (2000). IkappaB kinase alpha (IKKalpha) regulation of IKKbeta kinase activity. Mol. Cell. Biol. *20*, 3655–66.

Yost, C., Torres, M., Miller, J.R., Huang, E., Kimelman, D., and Moon, R.T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev. *10*, 1443–54.

Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell *91*, 243–252.